CRYOPRESERVATION OF GEMINAL VESICLE AND METAPHASE II STAGES BOVINE OOCYTES BY CRYOTOP AND SOLID SURFACE VITRIFICATION

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การแช่แข็งโอโอไซท์โคระยะ Germinal vesicle และ Metaphase II โดยวิธี Cryotop และ Solid Surface Vitrification

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วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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BOVINE/OOCYTE/VITRIFICATION/SOLID SURFACE VITRIFICATION/ CRYOTOP

Cryopreservation of bovine oocytes is important for the preservation and management of genetic resources. In this study Cryotop (CT) and solid surface vitrification (SSV) were used to vitrify germinal vesical (GV) and metaphase II (MII) stages bovine oocytes. The effects of pretreatment with cytochalasin B (CB), toxicity of cryoprotectant agents (CPA), and embryos development following *in vitro* fertilization (IVF) were evaluated. In the first experiment, the efficiency of CT and SSV methods and CB pretreatment were examined on GV stage oocytes. Cumulus oocyte complexes (COCs) were placed in 10% DMSO + 10% EG for 1 min and then exposed to 20% DMSO + 20% EG + 0.5 M sucrose for 30 sec. No significant differences were found among vitrified groups in maturation, cleavage and blastocyst rates after IVF. The GV stage bovine oocytes could be vitrified by CT and SSV methods with similar efficacy. Pre-treatment with CB did not increase the maturation and embryo development rates of vitrified oocytes. In the second experiment, the effects of CT and SSV vitrification methods, cryoprotectant (CPA) treatment on MII stage oocyte viability, IVF, pronucleus formation after IVF and subsequent *in vitro* development rate were assessed. After vitrified-warmed, lived oocytes were subjected to IVF and resultant embryos were cultured *in vitro*. After treatments the rates of lived oocytes were similar among the control, CPA, SSV and CT groups. There was no difference in rates of fertilization, pronuclear formation and monospermy among these groups. The cleavage rates for SSV (41.6%) and CT (53.2%) were significantly lower than

those in the control (65.9%) and CPA (61.3%) groups. The blastocyst rates in SSV (10.3%) and CT (12.8%) groups were not different, however, they were significantly lower than those in the control (36.4%) and CPA (24.8%) groups. Thus, MII stage bovine oocytes could be cryopreserved successfully using the CT and SSV methods.

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นุชจรินทร์ ศรีปัญญา : การแช่แข็งโอโอไซท์โครยะ Germinal vesicle และ Metaphase II โดยวิธี Cryotop และ Solid Surface Vitrification (CRYOPRESERVATION OF GEMINAL VESICLE AND METAPHASE II STAGES BOVINE OOCYTES BY CRYOTOP AND SOLID SURFACE VITRIFICATION) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 61 หน้า.

การแช่แข็งไข่โคมีความสำคัญในการเก็บรักษาและจัดการแหล่งพันธุกรรม การศึกษานี้ทำการแช่แข็งไข่โคในระยะ germinal vesical (GV) และระยะ metaphase II (MII) โดยวิธี Cryotop (CT) และ Solod Surface Vitrification (SSV) โดยทำการตรวจสอบผลของการนำไข่แช่ใน cytochalasin B (CB) และความเป็นพิษของน้ำยาแช่แข็ง (CPA) รวมถึงศึกษาอัตราการเจริญของตัวอ่อนหลังจากปฏิสนธิในหลอดแก้ว การทดลองแรก ทำการแช่แข็งไข่ระยะ GV ด้วยวิธี CT และ SSV โดยนำไข่ระยะ GV บ่มไว้ในน้ำยาซึ่งประกอบด้วย 10% DMSO + 10% EG เป็นเวลา 1 นาที จากนั้นนำไปไว้ในน้ำยาซึ่งประกอบด้วย 20% DMSO + 20% EG + 0.5 M Sucrose เป็นเวลานาน 30 วินาที พบว่าอัตราการเจริญของไข่ถึงระยะ MII การแบ่งตัวของตัวอ่อน และอัตราการเจริญของตัวอ่อนถึงระยะบลาสโตซีสหลังจากการปฏิสนธิในหลอดแก้วไม่มีความแตกต่างกันทางสถิติในกลุ่มที่ทำการแช่แข็ง และยังพบอีกว่าการแช่แข็งด้วยวิธี CT และ SSV มีประสิทธิภาพในการแช่แข็งไม่แตกต่างกัน การศึกษาที่สองนำไข่โครยะ MII มาทำการแช่แข็ง แล้วนำไปปฏิสนธิในหลอดแก้ว โดยตรวจสอบอัตราการรอดชีวิตหลังจากการละลาย การเกิดขึ้นของโปรนิวเคลียสหลังจากการปฏิสนธิในหลอดแก้ว และอัตราการเจริญของตัวอ่อน จากผลการทดลองพบว่า อัตราการรอดชีวิตของกลุ่มควบคุม กลุ่ม CPA กลุ่ม SSV และ กลุ่ม CT ไม่มีความแตกต่างกันทางสถิติ และยังพบอีกว่าอัตราการเกิดขึ้นของโปรนิวเคลียสหลังจากการปฏิสนธิในหลอดแก้วไม่แตกต่างกันในแต่ละกลุ่มการแบ่งตัวของไข่ที่แช่แข็งด้วยวิธี SSV (41.6%) และ CT (53.2%) มีอัตราต่ำกว่ากลุ่มควบคุม (65.9%) และกลุ่ม CPA (61.3%) อัตราการเจริญสู่ระยะบลาสโตซีสของไข่ที่แช่แข็งด้วยวิธี SSV (10.3%) และ CT (12.8%) ไม่มีความแตกต่างกัน แต่อย่างไรก็ตามอัตราการเจริญยังต่ำกว่ากลุ่มControl (36.4%) และ CPA (24.8%) การทดลองครั้งนี้สรุปได้ว่าการแช่แข็งไข่ระยะ MII ประสบผลสำเร็จได้ด้วยวิธี CT และ SSV

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ลายมือชื่อนักศึกษา นุชจรินทร์ ศรีปัญญา
ลายมือชื่ออาจารย์ที่ปรึกษา
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Nucharin Sripunya

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LIST OF ABBREVIATIONS

AI	=	artificial insemination
ART	=	assisted reproductive technique
COCs	=	cumulus-oocyte complexes
ICSI	=	intracytoplasmic sperm injection
SSV	=	solid surface vitrification
CT	=	cryotop
FDA	=	fluorescein diacetate
ICM	=	inner cell mass
TE	=	trophectroderm
IVF	=	*in vitro* fertilization
IVM	=	*in vitro* maturation
IVP	=	*in vitro* embryo production
GV	=	germinal vesicle
GVBD	=	germinal vesicle breakdown
MI	=	metaphase I
MII	=	metaphase II
CB	=	cytochalasin B
AI	=	anaphase I
TI	=	telophase I
OPS	=	open pulled straws
EM	=	electron microscope
DMSO	=	dimethylsulfoxide
EG	=	ethylene glycol

LIST OF ABBREVIATIONS (Continued)

LN_2	=	liquid nitrogen
BM	=	base medium
BSA	=	bovine serum albumin
mDPBS	=	modified Dulbecco`s phosphate buffer saline
PB	=	polar body
PI	=	propidium iodide
mSOFaa	=	modified oviduct synthetic fluid with amino acids

CHAPTER I

INTRODUCTION

1.1 Background

Assisted reproductive techniques, such as artificial insemination (AI), *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) have been used over the years in an effort to bypass critical fertilization steps, bypass breeding problems, and increase animal production efficiencies, overcome infertility and to propagate valuable genetics. Moreover, the use of ART procedures represent a plausible strategy to circumvent sexual incompatibility, eliminate the risk associated with animal transport and provide a method of using cryopreserved germ plasm to infuse genes from wild animals into captive breeding populations. But the limited number of bovine oocytes made it difficult to assess the success rate of ART in this trial.

Cryopreservation of oocytes from slaughtered animals has great value in increasing the availability of materials for basic research and their subsequent utilization for embryos production may provide an opportunity to replenish the endangered species gene banking and the genetic improvement of the livestock species.

To reduce the oocytes and embryos chilling injury, cytochalasin B (CB) have been reported to reduce damage to microtubules and enhance microtubule stabilization during vitrification as a cytoskeletal relaxant (Isachenko et al., 1998; Rho et al., 2002; Fujihira et al., 2004). However, effects of CB are controversial in many species and different procedures (Isachenko et al., 1998).

1.2 Research objectives

1.2.1 To examine the survival and maturation rates of germinal vesicle (GV) stage bovine oocytes vitrified by Cryotop and SSV methods.

1.2.2 To examine the development of IVF derived embryos from vitrified GV stage bovine oocytes using Cryotop and SSV methods.

1.2.3 To examine the viability of metaphase II (MII) stage bovine oocytes after vitrified by Cryotop and SSV methods.

1.2.4 To examine the IVF derived embryos development from vitrified MII stage bovine oocytes by using Cryotop and SSV methods.

1.3 Research hypothesis

1.3.1 The survival rates of oocytes after vitrified with Cryotop and SSV methods might be different**.**

1.3.2 The development of bovine embryos following IVF using oocytes vitrified with Cryotop and SSV might be different**.**

1.4 Scope and limitation of this study

Two vitrification techniques were used in this research. Fresh GV and MII stage bovine oocytes were vitrified by Cryotop and SSV methods. All of the survival by FDA staining. The survived oocytes were *in vitro* fertilized and cultured in SOFaa medium at 38.5°C in humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 for 7-9 days. Blastocysts were differential stained to determine trophectoderm (TE) and inner cell mass (ICM) cell numbers.

1.5 References

Isachenko, V., Soler, C., Isachenko, E., Perez-Sanchez, F., and Grishchenko, V. (1998). Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. **Cryobiology** 36: 250–253.

Rho, G.J., Kim, S., Yoo, J.G., Balasubramanian, S., Lee, H.J., and Choe, S.Y. (2002). Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. **Mol. Reprod. Dev.** 63: 464–470.

Fujihira, T., Kishida, R., and Fukui, Y. (2004). Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. **Cryobiology** 49: 286–290.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction of vitrification

Cryopreservation of bovine oocytes has been a great importance in the preservation of female genetic resources for further use. Cryopreserved oocytes can be used for nuclear transfer and genetic manipulation. The main problems with oocyte cryopreservation are concerned with the survival and fertilization rates. Most oocytes have been cryopreserved by either one of the two procedures: (1) equilibrium freezing which often referred to as "slow freezing" or (2) non-equilibrium freezing, which often referred to as "ultra-rapid cooling" or vitrification.

Vitrification is a process that produces a glasslike solidification of living cells that completely avoids ice crystal formation during cooling. And the vitrification process completely avoids ice crystal formation in cryopreserved cells during warming to recover the cells for biological applications. Vitrification could be beneficial compared with other freezing method because it does not need any expensive equipment and takes short time for cooling and warming. Vitrification is considered an attractive alternative to conventional freezing and has been applied successfully for the cryopreservation of embryos and the glass-like solidification by ultra-rapid cooling of mouse embryos, showing an alternative to traditional freezing methods to avoid chilling injury and ice crystal formation. There are two parameters for the success of cryopreservation: the speed of freezing (cooling rate),

and the effects of the dissolved substances, i.e. the concentration of the cryoprotective agents (CPA). The optimal cooling rate is the rates that permit most water to move out of the cells and freeze/vitrify extracellularly. To improve chances that the sample is surrounded with liquid and not vapor and to minimize the volume of the vitrification solution, special carriers are used during the vitrification process.

In vitrification techniques, the smallest possible volumes of vitrification solutions are used in specialized carrier or support devices, giving cooling rates approaching 10,000 ºC/min to 20,000 ºC/min. To improve chances that the sample is surrounded with liquid and not vapor, the sample size should be minimized and the duration of any vapor coat is reduced.

2.2 CPAs and vitrification methods

To achive high cooling rates the use of high concentration of the cryoprotectant solution, which depresses ice crystal formation are requires. Critical concentrations of the CPA are also required for vitrification. The minimal concentration can lead to either osmotic or chemical toxicity. Minimizing the toxicity of the CPAs resulting from the high cryoprotective concentration as well as reducing the cooling rate can be achieved by substituting an amino group for the hydroxyl (OH-) group of an alcohol, and increasing the hydrostatic pressure of the solution.

Successful vitrification for oocytes requires pretreatment with permeating CPA at a relatively low concentration before the final equilibration with the vitrification solution containing high concentrations of permeable and non-permeable CPAs. The low

intracellular CPA level of the pretreated oocytes would be concentrated to a higher level by severe dehydration in the vitrification solution during the equilibration period, which is the key factor to avoid intracellular ice formation by the subsequent cooling using liquid nitrogen (Hochi et al., 2004). The high CPA concentrations can be damaging to embryos, causing both biochemical alterations and lethal osmotic injury (Fahy et al., 1986). To minimize osmotic and toxic affects associated with concentration CPAs, toxicity can be reduced by combining two CPAs and adding precooled solutions. Conversely, it is possible to lower additives such as disaccharides (e.g. sucrose or trehalose) or high molecular weight molecules (e.g. Ficoll, polyvinylalcohol or polyvinyipyrrolidone) can significantly reduce the amount of permeable CPA required. The practical use of vitrification to preserve bovine oocytes is still limited since vitrified oocytes seem to display an impaired developmental competence after *in vitro* fertilization. Several methods had been developed to achieve rapid cooling by minimizing the volume of vitrification solution contain oocytes and embryos. The oocyte cryopreservation has been confirmed by the production of healthy calves born from frozen (Suzuki et al., 1996), vitrified (Vieira et al., 2002), immature bovine oocytes. This feasibility was further demonstrated by the birth of calves from cryopreserved embryos, derived from both frozen matured oocytes (Otoi et al., 1993) and from vitrified immature oocytes (Vieira et al., 2002). However, such as encouraging results, the cryopreservation of oocytes and *in vitro* embryo production (IVP) embryos is not yet an efficient established technology.

These methods differ in the CPA composition and treatment regimen and also in the way of cooling and storing the preserved specimen. The CPA can generally be divided into

two categories, permeating and nonpermeating. Permeating cryoprotectants are small molecules that readily penetrate the membranes of cells. One possible way to improve the cryotolerance of mature and immature oocytes may be the use of cytoskeleton stabilization such as cytochalasin B (CB) or taxol, which stabilizing the cytoskeleton system during vitrification and can also be beneficial for improving the post thaw survival and subsequent development of vitrified oocytes or embryos. The main damage observed during vitrification is an abnormal spindle configuration mainly due to the disorganization or disassembly of meiotic microtubules.

The CB as a cytoskeletal relaxant was considered to make the cytoskeletal elements less rigid (Fujihira et al., 2004). In mature oocytes, CB reduces damage to microtubules and may enhance spindle microtubules and may enhance stabilization of spindle microtubules during vitrification. In the case of germinal vesicle (GV) stage oocytes, no organized meiotic spindle is present, and this relaxant effect may preserve the functionality of the gap junctions faster and more uniform penetration of the cryoprotants on the vitrification of pig (Fujihira et al., 2004) and sheep (Silvestre et al., 2006) However, more studies are needed in order to increase oocytes or embryos survival after vitrification with these additives stabilization during vitrification of oocyte and embryo (Isachenko et al., 1997; Dobrinsky et al., 2000; Rho et al., 2004; Fujihira et al., 2004). The addition of taxol greatly improved post-thawing development of immature human oocytes (Fuchinoue et al., 2004), vitrified mouse and bovine oocytes (Park et al., 2001; Fuchinoue et al., 2004). Oocytes cryopreservation has been performed in several mammalian species. However, in spite of some successful report, the efficiency is still very low. It was demonstrated that

immature bovine oocytes are very sensitive to low temparatures of 4 or 0˚C, with a normal meiotic spindle formation that inhibit the normal development of oocytes after IVF. Mature bovine oocytes are sensitive to cooling, with occurrence of cytoskeleton rupture and meiotic spindle injuries. Immature oocytes had a germinal vesicle (GV) with a single condensed mass associated with the nucleolus, whereas those in germinal vesicle breakdown (GVBD) had an irregular envelope surrounding dispersed condensed chromatin. Metaphase I (MI) oocytes had a first metaphase plate. In oocytes at metaphase II (MII), the metaphase plate was located peripherally in the ooplasm and polar body in the perivitelline space.

The first successful cryopreserved *in vitro*-matured bovine oocytes by vitrification was reported by Martino and colleagues (1996) since then, there have been many tools designed for the special vitrification purpose. In 1998, Vajta and colleagues reported a novel way of vitrification using open pulled straws (OPS) for bovine embryos as well as oocytes. Later on the improved OPS methods using glass capillary (Kong et al., 2000; Hochi et al., 2000) and gel-loading tip (Tominaga et al., 2001) or the flexipet-denuding pipette (FDP) (Liebermann et al., 2002), micro drops (Papis et al., 2000; Atabay et al., 2004), electron microscope (EM) grids (Arav and Zeron, 1997; Hong et al., 1999; Park et al., 1999, 2000; Chung et al., 2000), hemi-straw system (Kuwayama and Kato, 2000; Vandervorst et al., 2001), solid surface vitrification (SSV) (Dinnyes et al., 2000), nylon mesh (Matsumoto et al., 2001), cryoloop (Lane et al., 1999) and Cryotop (Kuwayama and Kato, 2000) have been performed. Within those methods, SSV and Cryotop vitrifications

were reported to have a high efficiency to cryopreserve bovine oocytes (Dinnyes et al., 2000; Chian et al., 2004).

The Cryotop method is probably the latest minimum volume vitrification approach (Kuwayama and Kato 2000). A special tool consisting of a narrow, thin film strip (0.4 mm wide, 20 mm long 0.1 mm thick) attached to a hard plastic holder, has been developed. The Cryotop was originally used for vitrified human oocytes (Kuwayama and Kato 2000). Since then, Cryotop has been successfully used as device for vitrification of bovine oocytes (Chian et al., 2004), *in vitro* produced rabbit zygotes (Hochi et al., 2004), *in vitro* derived porcine embryos (Esaki et al., 2004), bovine and swamp buffalo blastocysts derived from somatic cell nuclear transfer (Laowtammathron et al., 2005), immature and *in vitro* matured horse oocytes (Bogliolo et al., 2006), *in vitro* matured swamp buffalo oocytes (Muenthaisong et al., 2007).

The SSV is a simple method that involves placing oocytes or embryos in a small amount of vitrification solution and directly drops on a metal surface cooled by liquid nitrogen. In 2000, Dinnyes and co-workers reported the successful cryopreservation of matured bovine oocytes using SSV. High survival rates after using SSV were reported for *in vitro* matured oocytes in goats (Begin et al., 2003). This methods have been used to achieve better result for the vitrification of embryos in species that are particularly susceptible to cryo-damage, including pig (Somfai et al., 2008), monkeys (Dinnyes et al., 2004), and buffalo (Gasparrini et al., 2007). In the present study, SSV was applied to cryopreserve GV and MII stage bovine oocytes for the first time, and its protective effect was evaluated, such as oocytes morphology, developmental potency and endocrine

function after cryopreservation. These methods have different cooling strategies and apply different cooling and carrier devices in GV and MII stages oocytes, it is necessary to determine the effects of cooling on bovine oocyte vitrification at both the GV and MII stages.

2.3 References

Arav, A., and Zeron, Y. (1997). Vitrification of bovine oocytes using modified minimum drop size technique (MDS) id effected by the composition and the concentration of the vitrification solution and by the cooling conditions. **Theriogenology** 47: 341 (abstract).

Atabay, E.C., Takahashi, Y., Katagiri, S., Nagano, M., Koga, A., and Kanai, Y. (2004). Vitrification of bovine oocytes and its application to intergeneric somatic cell nuclear transfer. **Theriogenology** 61: 15-23.

Begin, I., Bhatia, B., Baldassarre, H., Dinnyes, A., and Keefer, C.L. (2003). Cryopreservation of goat oocytes and *in vivo* derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. **Theriogenology** 59: 1839-1850.

Bogliolo, L., Ledda, S., Leoni, G., Naitana, S., and Moor, R.M. (2006). Activity of maturation promoting factor and mitogen-activated protein kinase after parthenogenetic activation of ovine oocytes. **Cloning** 2: 185-196.

Chian, R.C., Kuwayama, M., Tan, L., Tan, J., Kato, O., and Nagai, T. (2004). High survival rate of bovine oocytes matured *in vitro* following vitrification. **J. Reprod.**

Dev. 50: 685-696.

Chung, H.M., Hong, S.W., Lim, J.M., Lee, S.H., Cha, W.T., Ko, J.J., Han, S.Y., Choi, D.H., and Cha, K.Y. (2000). *In vitro* blastocyst formation of human oocytes obtained from unstimulated and stimulated cycles after vitrification at various maturational stages. **Fertil. Steril.** 73: 545-551.

Dinnyés, A., Dai, Y., Jiang, S., and Yang, X. (2000). High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. **Biol. Reprod.** 63: 513-518.

Dinnyés, A., Wei, S., Li, Y., Zheng, P., and Ji, W. (2004). First report on cleavage development following cryopreservation of adult and prepubertal rhesus monkey (*Macaca mulatana*) oocytes. **Reprod. Fert. Dev.** 16: 169.

Dobrinsky, J.R., Pursel, V.G., Long, C.R., and Johnson., L.A. (2000). Birth of Piglets After Transfer of Embryos Cryopreserved by Cytoskeletal Stabilization and Vitrification. **Biol. Reprod.** 62: 564-570.

Esaki, R., Ueda, H., Kurome, M., Hirakawa, K., Tomii, R., and Yoshioka, H. (2004). Cryopreservation of porcine embryos derived from *in vitro* matured oocytes. **Biol. Reprod.** 71: 432-437.

Fahy, G.M. (1986). **Vitrification: a new approach to organ cryopreservation.** In: Meryman HT (ed.), Transplantation: approaches to graft rejection, New York, Alan R Liss: 305-335.

Fuchinoue, K., Fukunaga, N., Chiba, S., Nakajo, Y., Yagi, A., and Kyono, K. (2004). Freezing of human immature oocytes using cryoloops with taxol in the vitrification

solution. **J. Assist. Reprod. Genet.** 21: 307-309.

Fujihira, T., Kishida, R., and Fukui, Y. (2004). Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. **Cryobiology** 49: 286-290.

Gasparrini, B., Attanasio, L., De Rosa, A., Monaco, E., Di Palo, R., and Campanile, G. (2007). Cryopreservation of *in vitro* matured buffalo (*Bubalus bubalis*) oocytes by minimum volumes vitrification methods. **Anim. Reprod. Sci.** 98: 335-342.

Hochi, S., Terao, T., Kamei, M., Kato, M., Hirabayashi, M., and Hirao, M. (2004). Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. **Theriogenology** 61: 267-75.

Hong, S.W., Chung, H.M., Lim, J.M., Ko, J.J., Yoon, T.K., Yee, B., and Cha, K.Y. (1999). Improved human oocyte development after vitrification: a comparison of thawing methods. **Fertil. Steril.** 72: 142-146.

Isachenko, V. (1997). Vitrification of porcine immature oocytes: new aspect. **Cryobiology** 1-2: 100-110.

Kong, I.K., Lee, S.I., Cho, S.G., Cho, S.K., and Park, C.S. (2000). Comparison of open pulled straws (OPS) vs glass micropipette (GMP) vitrification in mouse blastocysts. **Theriogenology** 53: 1817-1826.

Kuwayama, M., and Kato, O. (2000). All-round vitrification method for human oocytes and embryos. **J. Assist. Reprod. Genet.** 17: 477 (abstract).

Lane, M., Schoolcraft, W.B., and Gardner, D.K. (1999). Vitrificaiton of mouse and human blastocysts using a novel cryoloop container-less technique. **Fertil.**

Steril. 72: 1073-1078.

Laowtammathron, C., Lorthonpanich, C., Ketudat-Cairns, M., Hochi, S., and Parnpai, R. (2005). Factors affecting cryosurvival of nuclear- transferred bovine and swamp buffalo blastocysts: effects of hatching stage, linoleic acid-albumin in IVC medium and Ficoll supplementation to vitrification solution. **Theriogenology** 64: 1185-1196.

Leibermann, J.,Tucker, M.J., Graham, J., Taylor, H., Davis, D., and Levy, M.J. (2002). Blastocyst development after vitrification of multipronucleate zygotes using the flexipet denuding pipette (FDP). **RBM Online** 4: 148-152.

Martino, A., Songsasen, N., and Leibo, S.P. (1996). Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biol. Reprod.** 24: 1059- 1069.

Matsumoto, H., Jiang, J.Y., Tanaka, T., Sasada, H., and Sato, E. (2001). Vitrification of large quantities of immature bovine oocytes using nylon mesh. **Cryobiology** 42: 134-144.

Muenthaisong, S., Laowtammathro, C., Ketudat-Cairns, M., Parnpai, R., and Hochi, S. (2007). Quality analysis of buffalo blastocysts derived from oocytes vitrified before or after enucleation and reconstructed with somatic cell nuclei. **Theriogenology** 67: 893-900.

Otoi, T., Tachikawa, S., Kondo, S., Takagi, M., and Suzuki, T. (1993). Developmental capacity of bovine oocytes frozen in different cryoprotectants. **Theriogenology** 40: 801-807.

Papis, K., Shimizu, M., and Izaike, Y. (2000). Factors affecting the survivability of bovine

oocytes vitrified in droplets. **Theriogenology** 15: 651-658.

Park, S.E., Chung, H.M., Cha, K.Y., Hwang, W.S., Lee, E.S., and Lim, J. M. (2001). Cryopreservation of ICR mouse oocytes: improved post-thawed preimplantation development after vitrification using Taxol (TM), a cytoskeleton stabilizer. **Fertil. Steril.** 75: 1177-1184.

Park, S.P., Kim, E.Y., Kim, D.I., Park, N.H., Won, Y.S., Yoon, S.H., Chung, K.S., and Lim, J.H. (1999). Simple, efficient and successful vitrification of bovine blastocysts using electron microscopic grid. **Hum. Reprod.** 14: 2838-2843.

Park, S.P., Kim, E.Y., Oh, J.H., Nam, H.K., Lee, K.S., Park, S.Y., Park, E.M., Yoon, S.H., Chung, K.S., and Lim, J.H. (2000). Ultra rapid freezing of human multipronuclear zygotes using electron microscope grids. **Hum. Reprod.** 15: 1787-1790.

Rho, G.J., Lee, S.L., Kim, Y.S., Yeo, H.J., Ock, S.A., Balasubramanian, S., and Choe, S.Y. (2004). Intracytoplasmic sperm injection of frozen-thawed bovine oocytes and subsequent embryo development. **Mol. Reprod. Develop.** 68: 449-455.

Silvestre, M.A., Yániz, J., Salvador, I., Santolaria, P., and López-Gatius, F. (2006). Vitrification of pre-pubertal ovine cumulus–oocyte complexes: Effect of cytochalasin B pre-treatment. **Anim. Reprod. Sci.** 93: 176-182.

Somfai, T., Kashiwazaki, N., Ozawa M, Nakai, M., Maedomari, N., Noguchi, J., Kaneko, H., Nagai, T., and Kikuchi, K. (2008). Effect of centrifugation treatment before vitrification on the viability of porcine mature oocytes and zygotes produced *in vitro*. **J. Reprod. Dev.** 54: 149-155.

Suzuki, T., Boediono, A., Takagi, M., Saha, S., and Sumantri, C. (1996). Fertilization and

development of frozen-thawed germinal vesicle bovine oocytes by a one-step dilution method *in vitro*. **Cryobiology** 33: 515-524.

Tominaga, K., and Hamada, Y. (2001). Gel-loading tip as container for vitrification *in vitro*-produced bovine embryos. **Nat. Biotechnol.** 17: 1234-1236.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T., and Callesen, H. (1998). Open Pulled Straws (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. **Mol. Reprod. Dev.** 51: 53-58.

Vandervorst, M., Vanderzwalmen, P., Standaart, V., Debauche, C., Bertin, G., Zech, H., Zech, I., Bollen, N., van Roosendaal, E., and Schoysman, R. (2001). Blastocyst transfer after vitrification in a hemi-straw (HS) system. **Hum. Reprod.** 16 (abstract book 1): 153-154 (abstract 133).

Vieira, A.D., Mezzalira, A., Barbieri, D.P., Lehmkuhl, R.C., Rubin, M.I., and Vajta, G. (2002). Calves born after open pulled straw vitrification of immature bovine oocytes. **Cryobiology** 45: 91-94.

CHAPTER III

CRYOPRESERVATION OF GERMINAL VESICAL STAGE BOVINE OOCYTES: EFFECTS OF CYTOCHALASIN B PRETREATMENT ON THE EFFICIENCY OF CRYOTOP AND SOLID SURFACE VITRIFICATION METHODS

3.1 Abstract

The aim of the present study were to compare the efficiency of solid surface vitrification (SSV) and Cryotop (CT) vitrification methods, and pretreatment with cytochalasin B (CB) for cryopreservation of germinal vesicle (GV) stage bovine oocytes. The cumulus oocyte complexes (COCs) were placed in 10% dimethylsulfoxide (DMSO) + 10% ethylene glycol (EG) in TCM199 supplemented with 20% FBS for 1 min followed by 20% DMSO + 20% EG + 0.5 M sucrose in BM for 30 sec. CB pretreated [(+)CB] or non pretreated [(-)CB] COCs were vitrified either by SSV or CT. Surviving vitrified COCs were selected for IVM and IVF. No significantly difference was found among vitrified groups in maturation, cleavage and blastocyst rates after IVF. Similarly in solution exposure, after IVF, the cleavage and blastocyst rates among CB-exposed, FDA-exposed, CPA-exposed and CB+CPA-exposed were not differ with fresh control group. In conclusion, GV stage bovine oocytes could be vitrified by SSV and CT methods with similar efficacy. Pretreatment with CB did not increase the maturation and embryo development of vitrified oocytes.

3.2 Introduction

Cryopreservation of oocytes from slaughtered animals may enable a flexible utilization in time and space of live oocytes for research and animal production purposes. Likewise, subsequent use of cryopreserved oocytes for embryo production may provide an opportunity to replenish gene banks of endangered species and contribute to the genetic improvement of domestic animals.

Cryopreservation of immature oocytes would be a significant advance for basic research and commercial applications (Schroeder et al., 1990; Candy et al., 1994), which has been successful in mice (Eroglu et al., 1998), cattle (Suzuki et al., 1996), buffalo (Wani et al., 2004), porcine (Isachenko et al., 1998), human (Tucker et al., 1998). Recently, vitrification technique as an alternative method which has been used for oocyte cryopreservation rather than slow freezing technique (Vajta et al., 2000). The damages of oocytes or embryos caused by vitrification differ between species and may also depend on cryopreservation procedures (Chen et al., 2003). Two shortcomings of vitrification are the inevitably high concentrations of toxic cryoprotectants and chilling injury caused by sub-optimal cooling rates. To reduce the intracellular ice-crystal formation and chilling injury, it's feasible to increase the cooling rate. To do so, small volume samples are introduced to cooling device or directly place into liquid nitrogen (LN_2), such as SSV (Dinnyés et al., 2000), cryoloop (Lane et al., 1999), CT (Kuwayama and Kato 2000), microdrop (Papis et al., 2000), and electron microscope grid (Martino et al., 1996) techniques.

Pretreatment with the cytoskeletal relaxant, CB may also be used to reduce the chilling injury to oocytes and embryos. CB, a cytoskeletal relaxant has been reported to

improve survival and reduce damage to microtubules and enhance microtubule stabilization during vitrification (Isachenko et al., 1998; Rho et al., 2002; Dobrinsky et al., 2000; Fujihira et al., 2004). However, effects of CB are controversial in many species and different procedures (Ledda et al., 2001).

The current study was undertaken to compare the efficiency of SSV and CT vitrification methods for cryopreservation of immature bovine oocytes pretreated with or without CB and subsequent developmental ability to the blastocyst stage of vitrified oocytes after IVM and IVF were evaluated.

3.3 Materials and methods

3.3.1 Oocyte collection

Ovanies from crossbreed Holstein Friesian Cows were collected from slaughterhouses and kept in 0.9% NaCl solution during transportation to the laboratory at room temperature. The oocytes were collected by using a 10 mL syringe connected with 18G needle. Cumulus oocyte complexes (COCs) (Figure 1.1) were obtained by aspiration from 2-6 mm follicle in diameter and put into 15 mL conical tubes. All aspirated COCs with homogenous cytoplasm were partially denuded by repeat pipetting using a fine glass pipett in 0.2% hyaluronidase to be surrounded by 2 layers of cumulus cells and then used in the experiments.

3.3.2 Cytochalasin B treatment

The COCs were pretreated with 7.5 μg/mL CB in basic medium (BM), which was 25 mM Hepes TCM199 + 20% fetal bovine serum (FBS) for 15 min and then placed in equilibration and vitrification media containing 7.5 μg/mL CB and processed for

vitrification using SSV and CT methods. The viability of pretreated oocytes with CB was compared with non pretreated group as control. All of the vitrified oocytes were storaged in LN_2 for about 1-3 weeks.

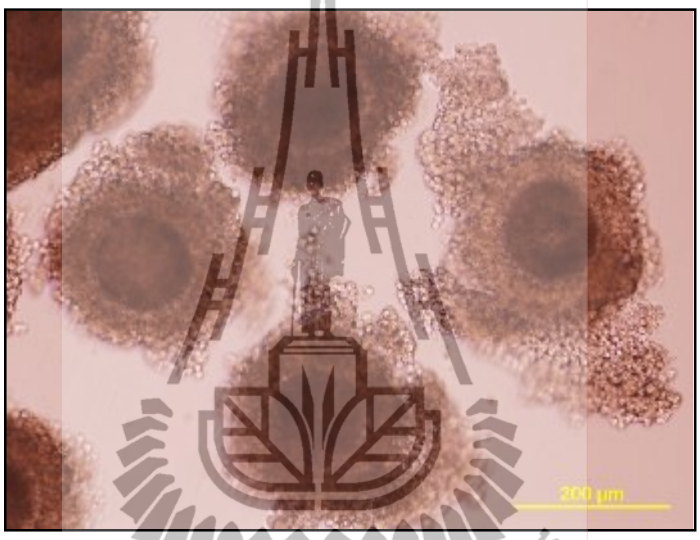


Figure 1.1 GV stage bovine oocytes after aspirated from ovaries.

3.3.3 Oocyte vitrification and warming

COCs were vitrified by CT method as described by Kuwayama and colleagues (2000). Briefly, group of 5 oocytes were washed in BM and then placed in BM containing 10% DMSO (v/v) and 10% EG (v/v) for 1 min. After this period they were transferred to BM containing 20% DMSO, 20% EG, and 0.5 M sucrose for 30 sec at 22–24°C. For the CT method, 5 oocytes in a volume <0.1 µL were loaded onto the surface of the film strip of each CT (Kitazato Supply Co., Tokyo, Japan), using a pulled Pasteur pipette. After loading, almost all of the solution was removed and the CT was directly immersed in LN_2, protected with a cap, and stored in LN_2. For the SSV method, groups of 5 oocytes in approximately 2 µL of vitrification solution were dropped onto chilled dry surface of an aluminum foil

floating on LN_2 using a glass capillary using cooled forceps. The vitrified droplets were then immersed in LN_2 for storage.

Vitrified oocytes were warmed by transferring microdrops/Cryotops into a warming solution (0.5 M sucrose in BM) at 38.5 ºC for 5 min. The oocytes were consecutively transferred for 5 min into each 500 µL droplets of BM supplemented with 0.5 M and 0 M sucrose, respectively. The oocytes were washed three times in BM at 38.5 ºC and returned into droplets of the IVM medium and were incubated for an additional 2 h at 38.5 ºC in humidified atmosphere of 5% CO_2 in air.

3.3.4 Evaluation of oocyte viability

After warming, the oocyte viability was evaluated by fluorescein diacetate (FDA) staining according to the method previously described by Mohr and Trounson (1980). Briefly, oocytes were treated with 2.5 µg/mL FDA in PBS supplemented with 5 mg/mL bovine serum albumin (BSA) at 38.5 ºC for 2 min in a dark room and then were washed three times in PBS supplemented with 5 mg/mL BSA and evaluated under an epifluorescence microscope (IX-71, Olympus, Tokyo, Japan) with UV irradiation using a U-MWIB3 filter with excitation wavelength of 460–495 nm and emission at 510 nm. Oocytes expressing a bright green fluorescence (Figure 1.2) were regarded as living ones and were subsequently used.

3.3.5 *In vitro* maturation

FDA positive COCs (20 per group) were cultured in 100 µL droplets of IVM medium covered with mineral oil in 60 mm plastic dishes (Nalge Nunc International, Roskilde, Denmark) a humidified atmosphere of 5% CO_2 in air with saturated humidity at 38.5°C for 22 h. The IVM medium consisting of TCM199 supplemented with 10% FBS,

50 IU/mL hCG, 0.02 AU/mL FSH and 1 µg/mL 17ß-estradiol, 10 ng/mL EGF, 100 µM cysteamine.

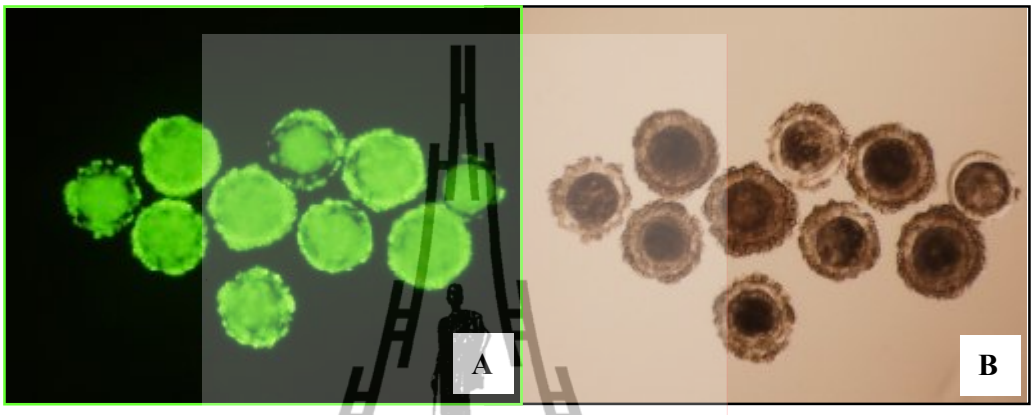


Figure 1.2 Oocytes stained by FDA and examine under fluorescent microscope. A: appearance of oocytes under fluorescent microscope (100x). B: appearance of oocytes under bright field fluorescent microscope (100x).

3.3.6 Evaluation of nuclear maturation

At 24 h of IVM, the cumulus cells were mechanically removed by repeated pipetting using a fine glass pipette in 0.2% hyarulonidase and were subsequently washed 4 times in modified Dulbecco`s phosphate buffer saline (mDPBS) supplement with 5 mg/mL BSA. The oocyte was evaluated as the method described by Somfai et al., (2008). Briefly, oocytes were mounted on glass slides and fixed on slides with acetic alcohol (acetic acid: ethanol, 1:3) for 3 days and stained with 1% (w/v) orcein in acetic acid for 5 min, then was rinsed in glycerol: acetic: water (1: 1: 3). After that, they were observed under a phase contrast microscope. Status of oocytes with a visible nuclear membrane was classified as GV stage. Oocytes beyond the GV stage were considered to have undergone germinal vesicle breakdown (GVBD). The absence of a visible nuclear membrane and the presence

of condensed chromatin were considered to indicate the metaphase I (MI) stage. Oocytes with a metaphase plate and one polar body (PB) were classified as stage MII.

3.3.7 *In vitro* fertilization

The frozen semen of fertile bull was thawed in 37°C water bath for 30 sec and then placed in the bottom of snap tube containing 2 mL BO solution (Brackett and Oliphant, 1975) supplemented with 10 mM caffeine, 10 mM hypotaurine for 30 min. The top of 1.8 mL of BO medium was then collected and placed in a conical centrifuge tube at 2,100×g for 7 min. After that, the pellets were re-suspended at the final concentrations of 3×10^6 spermatozoa/mL. 100 µL/drops of sperm suspension was prepared in 35 mm plastic dish and covered by mineral oil served as fertilization droplet. The IVM oocytes were washed twice in BO medium and then placed in fertilization droplets (20 oocytes/droplet) and cultured with spermatozoa for 10 h at 38.5°C in humidified atmosphere of 5% CO_2 in air with saturated humidity.

3.3.8 *In vitro* embryos culture

IVC was performed in 100 µL droplets of mSOFaa medium (Parnpai et al., 1999) supplemented with 3 mg/mL BSA and 100 µM cysteamine covered with paraffin oil. At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gentle pipetting with a fine glass pipette in preincubated IVC medium. Groups of 20 - 25 zygotes were placed in each culture drop and then were cultured at 38.5°C in humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 with saturated humidity for 9 days. Half of the medium was changed every two days. The day of IVF was considered as Day 0.

3.3.9 Differential staining

Blastocysts at day 7 to 9 were counter-stained to evaluate the inner cell mass (ICM) and trophectoderm (TE) cells by the method described by Thouas et al., (2001) with slight modifications. In brief, blastocysts were treated with 0.1 mg/mL propidium iodide (PI) and 0.2% Triton X-100 dissolved in mDPBS for 60 sec to permeabilize the membrane and stained the nuclei of the TE cells. The embryos were the treated with 25 µg/mL Hoechst 33342 dissolved in 99.5% ethanol for 5 min, and were mounted on glass slides in glycerol droplets. The ICM (blue) and TE cells (red) (Figure 1.3) were counted under a fluorescent microscope (IX-71, Olympus, Tokyo, Japan) at 330 to 380 nm, allowing determination of the total number of cells for blastocysts and the percentage of ICM cells based on the total number of cells.

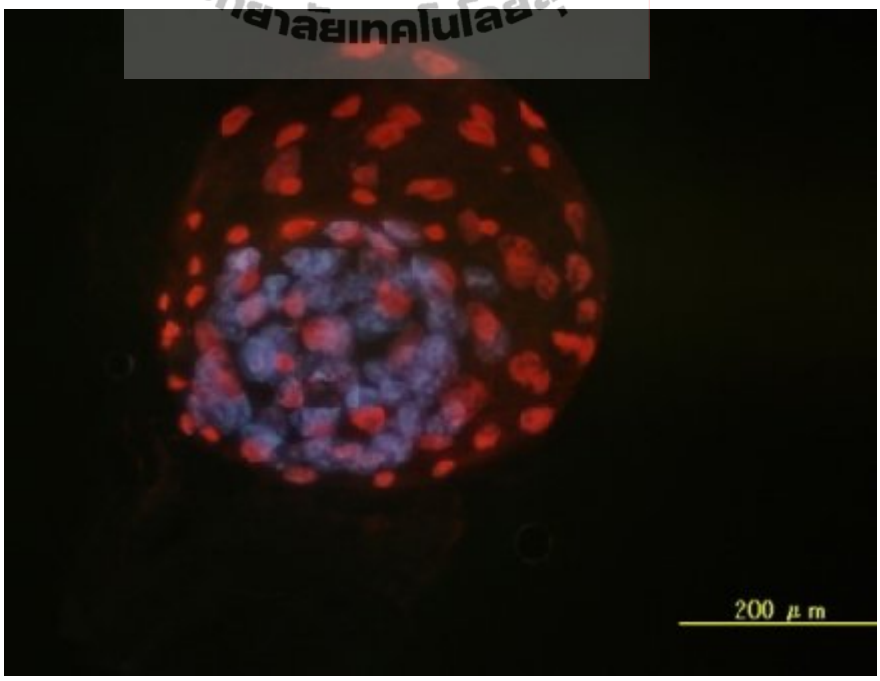


Figure 1.3 The embryonic cell nuclei were stained with Hoechst 33342.

3.3.10 Experimental design

Experiment 1: Toxicity of CPA

To investigate the toxicity of CB and the vitrification solution, partially denuded COCs were randomly separated into 5 groups: 1) Fresh COCs (Fresh control), 2) COCs exposed to FDA only (FDA-exposed), 3) COCs exposed to CB (CB-exposed), 4) COCs exposed to CPA (CPA-exposed), 5) COCs exposed to CB and CPA [CB(+)CPA-exposed], and oocyte viability in COCs was assessed by the FDA test except Fresh control group. COCs with surviving oocytes were subjected to IVM for 22 h. After IVM, oocyte viability was assessed again by the FDA test. Oocytes were then fixed to assess the nuclear maturation status.

Experiment 2: Effect of CB and vitrification devices on nuclear maturation status of vitrified oocytes

To assess the efficiency of the 2 vitrification devices [Cryotop (CT) and SSV] and the effects of CB used on nuclear maturation status after IVM. Partially denuded COCs with no pretreatment [CT(-)CB and SSV(-)CB groups,] or pretreated with 7.5 μg/mL CB for 15 min were vitrified using either the CT or the SSV method [CT(+)CB and SSV(+)CB groups,]. After warming, surving COCs (FDA-positive) were subjected to IVM for 22 h and then fixed to assess the nuclear maturation status.

Experiment 3: Effect of CB and CPA on development of oocytes after IVM and IVF

To assess the developmental ability of IVF embryos derived from CPA exposed GV stage oocytes. The COCs from Fresh control, FDA-exposed, CB-exposed,

CPA-exposed, and CB(+)CPA-exposed groups were warmed and cultured in IVM medium for 22 h. Subsequently, FDA-positive IVM oocytes were subjected to IVF.

Experiment 4: Effect of vitrification devices on development of oocytes after IVM and IVF

To assess the developmental ability of IVF embryos derived from vitrified GV stage oocytes. The COCs from CT(-)CB, SSV(-)CB, CT(+)CB and SSV(+)CB groups were warmed and cultured in IVM medium for 22 h. Subsequently, FDA-positive IVM oocytes were subjected to IVF.

3.3.11 Statistical analysis

Statistical analysis of data was evaluated by Completely Randomized Desigh (CRD) with Statistical Analysis System (SAS Inst. INC., Cary, N.C., USA). Analysis of Variance (ANOVA) and Comparison of means by Duncan's Multiple Range Test (DMRT) were analyzed. Different at $P<0.05$ were considered significant.

3.4 Results

3.4.1 Maturation status of oocytes exposed to CPA and CB

In vitro nuclear maturation status of CPA-exposed oocytes were summarized in Table. 3.1. There was no significant different in the rates of MI stage oocytes among FDA-exposed (15.7%), CB-exposed (12.8%), CPA-exposed (16.5%), CB(+)CPA-exposed (18.7%) and fresh control (12.1%) groups. Also there was no significant different in the rates of MII stage oocytes among FDA-exposed (80.7%), CB-exposed (82.8%), CPA-exposed (82.7%), CB(+)CPA-exposed (79.8%) and fresh control (86.4%) groups (Figure 1.4).

3.4.2 Effect of CB and vitrification devices on oocyte nuclear maturation

In vitro nuclear maturation status of vitrified oocytes was summarized in Table 3.2. At the end of IVM, there was no significant different in the rates of MI stage oocytes among Cryotop(-)CB (35.8%), Cryotop(+)CB (38.0%), SSV(-)CB (35.2%) and SSV(+)CB (31.8%) groups. Also, the rate of MII stage oocytes were similar among Cryotop(-)CB (58.3%), Cryotop(+)CB (57.0%), SSV(-)CB (59.7%) and SSV(+)CB (62.9%) groups. The vitrification methods and pre-treatment of oocytes with CB had no effect on the MII rates.

3.4.3 Effect of CPA and CB exposure on *in vitro* development of surviving oocytes after IVF

As shown in Table 3.3, there was no significant different among Fresh control, FDA-exposed, CB-exposed, CPA-exposed and CB(+)CPA-exposed groups in the cleavage and the blastocyst rates. Also, the blastocyst cell numbers showed no significant different among those groups. Pretreatment of oocytes with CB followed by CPA exposure did not improve the embryos development of GV stage bovine oocytes.

3.4.4 Effect of CB and vitrification devices on *in vitro* development of surviving oocytes after IVF

As shown in Table 3.4, there was not significant different among the 4 group of vitrified Cryotop(-)CB, Cryotop(+)CB, SSV(-)CB and SSV(+)CB in cleavage and blastocyst rates. The blastocyst cell numbers also showed no significant different among those groups. Pretreatment of oocytes with CB had no effect on the rates of blastocyst formation, regardless of the vitrification method used.

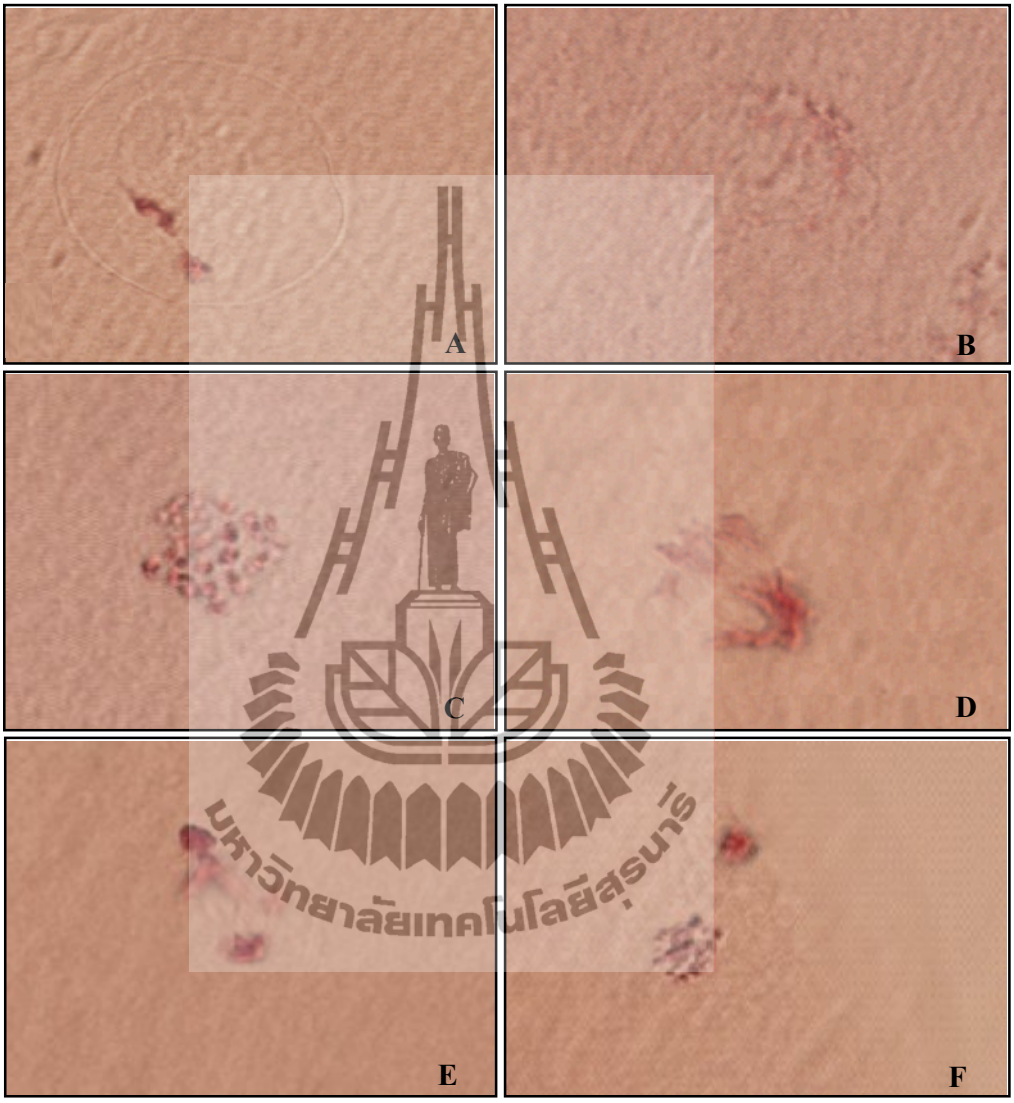


Figure 1.4 Nuclear appearance and classification of bovine GV oocytes; A: Germinal vesicle (GV), B: Germinal vesicle breakdown (GVBD), condensed chromosome and disappearance of the membrane, C: Metaphase I (MI), D: Anaphase I (AI), E: Telophase I (TI), F: Metaphase II (MII), (400x)

Table 3.1 *In vitro* nuclear maturation of GV stage bovine oocytes after CPA and CB exposure.

Groups	CB exposed	CPA exposed	FDA exposed	No. of oocytes cultured *in vitro*	No (%) of oocytes reached to						
					GV	GVBD	MI	AI	TI	MII	Chromosome not detected
Fresh control	-	-	-	140/140 (100.0)	0/140 (0.0)	0/140 (0.0)	17/140 (12.1)	0/140 (0.0)	1/140 (0.7)	121/140 (86.4)	1/140 (0.7)
FDA-exposed	-	-	+	140/140 (100.0)	1/140 (0.7)	2/140 (1.4)	22/140 (15.7)	1/140 (0.7)	1/140 (0.7)	113/140 (80.7)	0/140 (0.0)
CB-exposed	+	-	+	140/141 (99.3)	0/140 (0.0)	1/140 (0.7)	18/140 (12.9)	0/140 (0.0)	0/140 (0.0)	116/140 (82.9)	1/140 (0.7)
CPA-exposed	-	+	+	139/141 (98.6)	0/139 (0.0)	0/139 (0.0)	23/139 (16.5)	0/139 (0.0)	0/139 (0.0)	115/139 (82.7)	1/139 (0.7)
CB(+)CPA-exposed	+	+	+	139/140 (99.3)	0/139 (0.0)	0/139 (0.0)	26/139 (18.7)	1/139 (0.7)	0/139 (0.0)	111/139 (79.8)	1/139 (0.7)

Seven replications were performed. CB : Cytochalasin B; CPA: Cryoprotectant; FDA: Fluorescein diacetate; GV: germinal vesicle; GVBD: germinal vesicle breakdown; MI: metaphaes I; AI: anaphase I; TI: telophase I; MII: metaphase II

Table 3.2 *In vitro* nuclear maturation of GV stage bovine oocytes after vitrification.

Groups	CB exposed	FDA exposed	No. of oocytes cultured *in vitro*	No (%) of oocytes reached to						
				GV	GVBD	MI	AI	TI	MII	Chromosome not detected
Cryotop(-)CB	-	+	151/158	4/151	2/151	54/151	0/151	0/151	88/151	3/151
			(95.5)	(2.6)	(1.3)	(35.8)	(0.0)	(0.0)	(58.3)	(2.0)
Cryotop(+)CB	+	+	142/156	4/142	0/142	54/142	1/142	0/142	81/142	2/142
			(91.0)	(2.8)	(0.0)	(38.0)	(0.7)	(0.0)	(57.0)	(1.4)
SSV(-)CB	-	+	139/150	5/139	0/139	49/139	2/139	0/139	83/139	0/139
			(92.6)	(3.6)	(0.0)	(35.3)	(1.4)	(0.0)	(59.7)	(0.0)
SSV(+)CB	+	+	132/147	5/132	1/132	42/132	0/132	1/132	83/132	1/132
			(89.8)	(3.8)	(0.8)	(31.8)	(0.0)	(0.8)	(62.9)	(0.8)

Six replications were performed. CB: Cytochalasin B; CPA: Cryoprotectant ; FDA: Fluorescein diacetate; GV: germinal vesicle;

GVBD: germinal vesicle breakdown; MI: metaphaes I; AI: anaphase I; TI: telophase I; MII: metaphase II

Table 3.3 *In vitro* embryo development of solution exposed GV stage bovine oocytes following IVF.

Groups	CB exposed	CPA exposed	FDA exposed	No.of IVF	No. (%) of oocytes developed to				No.of blastocysts evaluated	No. (%) of nuclei ± SEM		
					Cleavage	8-C	Morula	Blastocysts		TE	ICM	ICM ratio
Fresh control	-	-	-	125	65/125 (52.0)	55/125 (44.0)	34/125 (27.2)	30/125 (24.0)	24	23.8 ± 2.1	31.6 ± 0.6	22.1 ± 0.4
FDA-exposed	-	-	+	125	67/125 (53.6)	49/125 (39.2)	31/125 (24.8)	24/125 (19.2)	21	94.9 ± 1.9	47.8 ± 1.1	33.0 ± 0.6
CB-exposed	+	-	+	125	59/125 (47.2)	45/125 (36.0)	27/125 (21.6)	22/125 (17.6)	18	104.5 ± 2.4	38.0 ± 0.9	28.2 ± 0.5
CPA-exposed	-	+	+	125	64/125 (51.2)	50/125 (40.0)	32/125 (25.6)	25/125 (20.0)	18	100.8 ± 2.5	36.1 ± 0.9	28.3 ± 0.7
CB(+)CPA-exposed	+	+	+	125	67/125 (53.6)	49/125 (39.2)	27/125 (21.6)	22/125 (17.6)	12	99.3 ± 2.5	33.7 ± 1.6	25.1 ± 0.8

Five replications were performed.

CB: Cytochalasin B; CPA: Cryoprotectant; FDA: Fluorescein diacetate.

Table 3.4 *In vitro* embryo development of vitrified GV stage bovine oocytes following IVF.

Groups	CB exposed	FDA exposed	No.of IVF	No. (%) of oocytes developed to				No.of blastocysts evaluated	No. (%) of nuclei ± SEM		
				Cleavage	8-C	Morula	Blastocysts		TE	ICM	ICM ratio
Cryotop(-)CB	-	+	155	56/155 (36.1)	22/155 (14.2)	13/155 (8.4)	9/155 (5.8)	6	102.7 ± 6.9	42.0 ± 2.8	30.4 ± 2.0
Cryotop(+)CB	+	+	149	35/149 (23.5)	21/149 (14.1)	11/149 (7.4)	10/149 (6.7)	5	65.0 ± 5.4	26.4 ± 2.0	30.6 ± 2.2
SSV(-)CB	-	+	151	52/151 (34.4)	26/151 (17.2)	10/151 (6.6)	6/151 (4.0)	6	98.8 ± 7.4	32.6 ± 3.4	26.6 ± 2.3
SSV(+)CB	+	+	146	38/146 (26.0)	22/146 (15.1)	9/146 (6.2)	9/146 (6.2)	7	86.7 ± 6.4	31.8 ± 1.5	28.2 ± 0.9

Five replications were performed.

CB: Cytochalasin B; CPA: Cryoprotectant; FDA: Fluorescein diacetate.

3.5 Discussion

The present study evaluated the effects of different cryopreservation devices (CT and SSV) and CB on the maturation rates, embryos development after IVM and IVF with vitrified GV stage bovine oocytes.

The major finding of this study is that GV stage bovine oocytes could be successfully cryopreserved by vitrification with CT and SSV methods, as indicated by their development to the blastocyst stage after IVM and IVF. However, the blastocyst formation rates were extremely low. Changes of cell volume and intracellular CPA concentrations are more severe in MII than GV bovine oocytes during CPA addition and dilution process, which make it more sensitive (Yang et al., 2010). The low survivability in GV stage oocytes may be due to lower membrane permeability of the plasma membrane to cryoprotectant changes. It is possible, therefore, that during maturation culture, GV stage oocytes surviving sublethal damage induced by cryopreservation may recover.

Vitrification was carried out by SSV and CT. The SSV methods using large volume of vitrification solution lead to the decreasing of cooling and warming rates (Pollard et al., 1994), and modifications that aimed to circumvent the cellular disruptions during vitrification and might lead to the improved survival and embryonic development. Up to 62.0% of the GV stage oocytes reached MII stage following SSV and up to 6.0% of the GV stage oocytes developed to blastocyst stage after IVM and IVF. Therefore, SSV was effective for the cryopreservation of bovine oocytes. In the present study, CT gave the similar MII, cleavage and blastocyst rates compared with SSV method. Using this method cryopreserved cells are less likely to experience solution effects and intracellular ice formation compared with the traditional method of slow cooling (Fahy et al., 1984).

On the other hand, due to the high concentrations of CPA, the cells may be exposed to detrimental osmotic effects. One essential factor in cryosurvival is permeation of a certain amount of toxic cryoprotectant into the oocytes. Therefore, the period of exposure

to CPAs before plunging into LN_2 is critical and should not be extended. High concentration of CPAs used for vitrification has been proven to be toxic to cells (Fuller et al., 2004; Kuwayama et al., 2005). The result showed that there was no different among groups in the number of oocytes classified meiotic stages of maturation following vitrification and thawing without cooling used the GV stage bovine oocytes groups after vitrification that reached to MII stage. Improved blastocyst yield and quality were achieved due to stimulating of the pattern of protein indicated in bovine oocytes.

In a previous study, vitrification of buffalo oocytes using 20% EG + 20% DMSO resulted in 11.5% morula and 4.3% blastocyst development (Gautam et al., 2008). In other reports, bovine (Vieira et al., 2007) and buffalo (Manjunatha et al., 2008) blastocysts vitrified in 20% EG + 20% DMSO showed better hatching rates than those vitrified in 40% EG. Similar reports also indicated that EG with DMSO when used as CPAs mixture may have some advantages over solutions having single penetrating CPAs (Vajta et al., 1999). EG has been considered to be less toxic and with high osmotic ability to the oocytes and embryos (Kasai et al., 1996). The beneficial effects of cumulus cells on oocytes survival rate after freezing-thawing have been reported and have suggested that oocytes survival rates following vitrification were not affected by the presence of cumulus cells. The toxicity test in this study showed that CPA regimen is suitable for GV stage bovine oocytes.

The cytoskelelon in first meiotic division of immature oocytes is particularly vulnerable to cryodamage, whereas matured oocytes display a more flexible cytoskeleton. The numerically lower maturation rate in the vitrified GV stage oocytes compared to the non-vitrified oocytes may be due to chromosomal aberrations that would have taken place during vitrification to alteration in the meiotic spindles (Carroll et al., 1993). High cooling rate could prevent the sensitivity of the cell from chilling injury (Martino et al., 1996). Thus, successful vitrification requires high concentrations of permeable cryoprotectants

and a very rapid cooling rate, which can be provided by both the CT and SSV methods. CT method applies a thin plastic sheet on which oocytes/embryos are placed with a minimum amount of vitrification solution before plunging into LN_2. On the other hand, the SSV method achieves a high cooling rate by using a combination of microdrops and improved heat exchange by direct contact with a dry metal surface cooled by LN_2 (Dinnyés et al., 2000).

Similar fertilization and nuclear maturation rates were observed after IVM among CB-exposed, CPA-exposed, CB+CPA-exposed and FDA-exposed, groups. Also in vitrification groups, similar results were found after IVM and IVF. These results suggested that the solution control treatments, both cooling methods and the warming regimen used in the present study did not cause such phenomena in GV stage bovine oocytes. These results indicated that the cooling strategies of the SSV and CT vitrification methods are equally effective for cryopreservation of GV stage bovine oocytes. In this respect, both of these cooling methods can be recommended for cattle oocyte vitrification.

Similar to previous studies of cattle and pig oocytes (Dinnyés et al., 2000), treatment of GV stage oocytes with cryoprotectants without vitrification according to the SSV protocol did not influence fertilization and *in vitro* development. Taken together with the high survival rates, this suggests that the impaired developmental ability of vitrified oocytes was caused by sub-lethal damage that could be mainly related to the cooling and/or warming procedure. Such sub-lethal damage may include disruption of the meiotic spindle and other cytoskeletal elements (Chen et al., 2003; Albarracín et al., 2005), damage and dysfunction of organelles such as mitochondria or endoplasmic reticulum (Rho et al., 2002; Lowther et al., 2009) or degradation of cytoplasmic mRNA levels (Succu et al., 2008) which have been reported to occur in cryopreserved mammalian oocytes.

Cytochalasin B is a cell-permeable mycotoxin that inhibits microfilament polymerization and therefore organelle movements and nuclear extrusion in cells. CB has

been reported as a cytoskeletal relaxant to reduce microtubular injury during vitrification (Rho et al., 2002; Fujihira et al., 2004). However, there are controversial reports on the effect of CB for oocytes and embryos vitrification depending on the species and the procedure used. In pigs CB pretreatment has been reported to improve cryosurvival of oocytes and embryos (Isachenko et al., 1998; Dobrinsky et al., 2000). On the other hand CB pretreatment has been found to be ineffective for the improvement of vitrification efficacy of bovine matured oocytes (Mezzalira et al., 2002) and ovine immature oocytes (Silvestre et al., 2006) in accordance with our present results.

This study evaluated the effect of adding CB microfilament inhibitor stabilizing microtubules, for protection of microchondria and the ability of bovine oocytes from normal spindles after cryopreservation. The results suggest that microtubule is closely associated with reconstruction and proper positioning of chromatin during meiotic maturation in bovine oocytes. Rewarming may then lead to membrane fusion, cortical granule enzyme relate and premature zona hardening, thus as possible blocking fertilization completely, the result in a reduced cleavage rate after insemination (Lim et al., 1991). Further improvements of the present vitrification protocols will be necessary to avoid such damage.

3.6 Conclusion

In conclusion, this result indicate that both CT and SSV vitrification methods could yield similar maturation, cleavage and blastocyst rates of bovine GV stage bovine oocytes after IVM and IVF. At higher rates than GV partially-denuded oocytes using the Cryotop method (Zhou et al., 2010). Pretreatment with CB did not increase the maturation and embryo development of vitrified oocytes. However, further studies are necessary to improve the developmental competence of cryopreserved bovine oocytes.

3.7 References

AlbarracÍn, J.L., Morató, R., Izquierdo, D., and Mogas, T. (2005). Vitrification of calf oocytes: Effects of maturation stage and prematuration treatment on the nuclear and cytoskeletal components of oocytes and their subsequent development. **Mol. Reprod. Dev.** 72: 239-249.

Candy, C.J., Wood, M.J., Whittingham, D.G., Merriman, J.A., and Choudhury, N. (1994). Cryopreservation of immature mouse oocytes. **Hum. Reprod.** 9: 1738-1742.

Carroll, J., Wood, M.J., and Whittingham, D.G. (1993). Normal fertilization and development of frozen thawed mouse oocytes: Protective action of certain macromolecules. **Biol. Reprod.** 48: 606-612.

Chen, S.U., Lien, Y.R., Chao, K.H., Ho, H.N., Yang, Y.S., and Lee, T.Y. (2003). Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing-a review article. **Mol. Cell Endocrinol**. 202: 101–107.

Dinnyes, A., Dai, Y., Jiang, S., and Yang, X. (2000). High developmental rates of vitrified bovine oocytes follwing parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. **Biol. Reprod**. 63: 513-518.

Dobrinsky, J.R., Pursel, V.G., Long, C.R., and Johnson, L.A. (2000). Birth of piglets after transfer of embryos cryopreserved by cytoskeletal stabilization and vitrification. **Biol. Reprod**. 62: 564-570.

Eroglu, A., Toth, T.L., and Toner, M. (1998). Alterations of the cytoskeleton and polyploidy induced by cryopreservation of metaphase II mouse oocytes. **Fertil. Steril**. 69: 944-957.

Fahy, G.M., McFralene, D.R., Angell, C.A. and Merymen, H.T. (1984). Vitrification as an approach to cryopreservation. **Cryobiology** 21: 407-426.

Fuchinoue, K., Fukunaga, N., Chiba, S., Nakajo, Y., Yagi, A., and Kyono, K. (2004).

Freezing of human immature oocytes using cryoloops with taxol in the vitrification solution. **J. Assist. Reprod. Genet.** 21: 307-309.

Fujihira, T., Kishida, R., and Fukui, Y. (2004). Developmental capacity of vitrified immature porcine oocytes following ICSI: effects of cytochalasin B and cryoprotectants. **Cryobiology** 49: 286-290.

Fuller, B.J. (2004). Cryoprotectants: the essential antifreezes to protect life in the frozen state. **Cryo. Letters.** 25: 375-378.

Gautam, S.K., Verma, V., Palta, P., Chauhan, M.S., and Manik, R.S. (2008). Effect of type of cryoprotectant on morphology and developmental competence of *in vitro*-matured buffalo (*Bubalus bubalis*) oocytes subjected to slow freezing or vitrification, **Reprod. Fertil. Dev**. 20: 490-496.

Isachenko, V., Soler, C., Isachenko, E., Perez-Sanchez, F., Grishchenko, V. (1998). Vitrification of immature porcine oocytes: effects of lipid droplets, temperature, cytoskeleton, and addition and removal of cryoprotectant. **Cryobiology** 3: 250-253.

Kasai, S., Sugimura, K., Matsumoto, K., Nishi, N., Kishimoto, T., and Nakamura, T. (1996). Hepatocyte growth factor is a paracrine regulator of rat prostate epithelial growth. **Biochem. Biophys. Res. Commun.** 228: 646-652.

Kuwayama, M., and Kato, O. (2000). All-round vitrifcation method for human oocytes and embryos. **J. Assist. Reprod. Genet.** 17: 477.

Kuwayama, M., Vajta, G., Ieda, S., Kato, O. (2005). Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. **Reprod. Biomed. Online.** 11: 608-614.

Lane, M., Bavister, B.D., Lyons, E.A., and Forest, K.T. (1999). Containerless vitrification of mammalian oocytes and embryos. **Nat. Biotechnol**. 17: 1234-1236.

Ledda, S., Leoni, G., Bogliolo, L., and Naitana, S. (2001). Oocytes cryopreservation and ovarian tissue banking. **Theriogenology** 55: 1359–1371.

Lim, J.M., Fukui, Y., and Oho, H. (1991). The post-thaw developmental capacity of frozen bovine oocytes following *in vitro* matureation and fertilization. **Theriogenology** 35: 1225-1235.

Lowther, K.M., Weitzman, V.N., Maier, N., and Mehlmann, L.M. (2009). Maturation, fertilization, and the structure and function of the endoplasmic reticulum in cryopreserved mouse oocytes. **Biol. Reprod.** 81: 147-154.

Manjunatha, B.M., Gupta, P.S., Ravindra, J.P., Devaraj, M., and Nandi, S. (2008). *In vitro* embryo development and blastocyst hatching rates following vitrification of river buffalo embryos produced from oocytes recovered from slaughterhouse ovaries of live animals by ovum pick-up. **Anim. Reprod. Sci.** 104: 419-426.

Martino, A., Songsasen, N., and Leibo, S.P. (1996). Development into blastoysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biol. Reprod.** 54: 1059-1069.

Mezzalira, A., Vieira, A.D., Barbieri, D.P., Machado, M.F., Thaler, N.A., Bernardi, M.L., Silva, C.A.M., and Rubin, M.I.B. (2002). Vitrification of matured bovine oocytes treated with cytochalasin B. **Theriogenology** 57: 472 (Abstract).

Mohr, L.R., and Trounson, A.O. (1980). The use of fluorescein diacetate to assess embryo viability in the mouse. **J. Reprod. Fertil.** 58: 189–196.

Papis, K., Shimizu, M., and Izaike, Y. (2000). Factors affecting the survivability of bovine oocytes vitrified in droplets. **Theriogenology** 15: 651-658.

Parnpai, R., Tasripoo, K., and Kamonpatana, M. (1999). Development of cloned swamp buffalo embryos derived from fetal fibroblast: Comparison *in vitro* cultured with or without buffalo and cattle epithelial cells. **Buffalo. J.** 15: 371–384.

Pollard, J.W., and Leibo, S.P. (1994). Chilling sensitivity of mammalian embryos. **Theriogenology** 41: 101-106.

Rho, G.J., Kim, S., Yoo, J.G., Balasubramanian, S., Lee, H.J., and Choe, S.Y. (2002).

Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. **Mol. Reprod. Dev.** 63: 464–470.

Schroeder, A.C., Champlin, A.K., Mobraaten, L.E., and Eppig, J.J. (1990). Development capacity of mouse oocytes cryopreserved before and after maturation *in vitro*. **J. Reprod. Fertil.** 89: 43-50.

Silvestre, M.A., Yániz, J., Salvador, I., Santolaria, P., and López-Gatius, F. (2006). Vitrification of pre-pubertal ovine cumulus–oocyte complexes: Effect of cytochalasin B pre-treatment. **Anim. Reprod. Sci.** 93: 176-182.

Somfai, T., Kashiwazaki, N., Ozawa, M., Nakai, M., Maedomari, N., Noguchi , J., Kaneko, H., Nagai, T., and Kikuchi, K. (2008). Effect of centrifugation treatment before vitrification on the viability of porcine mature oocytes and zogotes produced *in vitro*. **Reproduction** 54: 149-155.

Succu, S., Bebbere, D., Bogliolo, L., Ariu, F., Fois, S., Leoni, G.G., Berlinguer, F., Naitana, S., and Ledda, S. (2008). Vitrification of *in vitro* matured ovine oocytes affects *in vitro* pre-implantation development and mRNA abundance. **Mol. Reprod. Dev.** 75: 538–545.

Succu, S., Leoni, G.G., Berlinguer, F., Madeddu, M., Bebbere, D., Mossa, F., Bogliolo, L., Ledda, S., and Naitana, S. (2007). Effect of vitrification solutions and cooling upon *in vitro* matured prepubertal ovine oocytes. **Theriogenology** 68: 107-114.

Suzuki, T., Boediono, A., Takagi, M., Saha, S., and Sumantri, C. (1996). Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by a one-step dilution method *in vitro*. **Cryobiology** 33: 515–524.

Thouas, G.A., Korfiatis, N.A., French, A.J., Jones, G.M., and Trounson, A.O. (2001). Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. **Reprod. Biomed. Online.** 3: 25-29.

Tucker, W.G., Morton, P.C., and Massey, J.B. (1998). Birth after cryopreservation of

immature oocytes with subsequent *in vitro* maturation. **Fertil. Steril.** 70: 578-579.

Vajta, G. (2000). Vitrification of the oocytes and embryos of domestic animals. **Anim. Reprod. Sci.** 60-61: 357-364.

Vajta, G., Rindom, N., Peura, T.T., Holm, P., Greve, T., and Callesen, H. (1999). The effect of media, serum and temperature on *in vitro* survival of bovine blastocysts after open pulled straw (OPS) vitrification. **Theriogenology** 5: 939-948.

Vieira, A.D., Forell, F., Feltrin, C., and Rodrigues, J.L. (2007). In-straw cryoprotectant dilution of IVP bovine blastocysts vitrified in hand-pulled glass micropipettes. **Anim. Reprod. Sci.** 99: 377-383.

Wani, N.A., Misra, A.K., and Maurya, S.N. (2004). Maturation rates of vitrified-thawed immature buffalo (*Bubalus bubalis*) oocytes: effect of different types of cryoprotectants. **Anim. Reprod. Sci.** 84: 327-335.

Yang, X., Al Naib, A., Sun, D.W., and Lonergan, P. (2010). Membrane permeability characteristics of bovine oocytes and development of a step-wise cryoprotectant adding and diluting protocol. **Cryobiology** 61: 58-65.

Zhou, X.L., Naib, A. Al., Sun, D.W., and Lonergan, P. (2010). Bovine oocyte vitrification using the Cryotop method: Effect of cumulus cells and vitrification protocol on survival and subsequent development. **Cryobiology** 61: 66-72.

CHAPTER IV

A COMPARISON OF CRYOTOP AND SOLID SURFACE VITRIFICATION METHODS FOR THE CRYOPRESERVATION OF IN VITRO MATURED BOVINE OOCYTES

4.1 Abstract

The aim of the present study was to compare the efficacies of the cooling systems of the solid surface (SSV) and Cryotop vitrification methods for the cryopreservation of bovine oocytes at the metaphase II (MII) stage. The effects of vitrification and cryoprotectant treatment on oocyte viability, *in vitro* fertilization (IVF), pronucleus formation after IVF and subsequent *in vitro* development were assessed. *In vitro* matured (IVM) bovine oocytes were subjected to equilibration and vitrification solutions according to the SSV method, and then the oocytes were vitrified either by dropping on a cold dry metal surface (SSV group) or by plunging into liquid nitrogen on Cryotop sheets (Cryotop group). Warming was conducted according to the SSV method. Some oocytes were subjected to cryoprotectants and warming regimen without cooling (CPA group). After warming the live/dead status of oocytes was evaluated by fluorescein diacetate staining. Live oocytes were subjected to IVF and resultant embryos were cultured *in vitro*. After treatment the rates of live oocytes were similar among the Control, CPA, SSV and Cryotop groups. There was no difference in rates of fertilization, pronuclear formation and monospermy among these groups. The cleavage rates for SSV (41.6%) and Cryotop

(53.2%) groups were significantly lower than those in the Control (65.9%) and CPA (61.3%) groups. The blastocyst rates in SSV (10.3%) and Cryotop (12.8%) groups were not significantly different; however, they were lower than those in the Control (36.4%) and CPA (24.8%) groups. The inner cell mass, trophectoderm and total cell numbers in the blastocysts were not significantly different among the Control, CPA, SSV and Cryotop groups. Our results indicate that *in vitro* matured bovine oocytes could be cryopreserved successfully using the cooling systems of both Cryotop and Solid Surface Vitrification methods with similar efficacy without reducing survival rates.

4.2 Introduction

In general, oocytes are more susceptible to cooling damage than zygotes because during the cooling metaphase spindle microtubule integrity is disrupted and the high concentration of cryoprotectants affects oocytes during equilibration (Chen et al., 2003). Also, differences in the membrane structures seem to make oocytes more sensitive to chilling compared to zygotes (Ghetler et al., 2005). Although the toxicity of cryoprotectants can be minimized by increasing the cooling rate (Martino et al., 1996), other side effects of cooling and cryoprotectants such as zona hardening and parthenogenetic activation may affect fertilization results and cause low development rates to the blastocyst stage (Carroll et al., 1990: Somfai et al., 2007). Successful vitrification of oocytes requires a pretreatment with permeating CPAs at a relatively low concentration before the final equilibration with the vitrification solution containing high concentrations of permeating and non-permeable CPAs. The low intracellular CPA level of the pretreated oocytes would be concentrated to a higher level by severe dehydration in the vitrification solution during the equilibration period, which is a key factor to avoid intracellular ice formation by the subsequent cooling using liquid nitrogen (Hochi et al., 2004). The

practical use of vitrification to preserve bovine oocytes is still limited since vitrified oocytes seem to display an impaired developmental competence after *in vitro* fertilization. Several methods had been developed to achieve rapid cooling by minimizing the volume of vitrification solution containing oocytes and embryos such as vitrification with electron microscope grids (Martino et al., 1996), glass capillaries (Hochi et al., 1994), open pulled straws (Vajta et al., 1998), cryoloops (Lane et al., 1999), Cryotops (Kuwayama et al., 2000) and solid-surface vitrification (SSV) (Dinnyés et al., 2000). These methods differ in CPA composition and treatment regimen and also in the methods for cooling and storing the preserved specimens. Two methods, the SSV and Cryotop vitrifications were reported to have a high efficiency to cryopreserve bovine oocytes (Dinnyés et al., 2000; Chian et al., 2004). These methods have different cooling strategies and apply different cooling and carrier devices. The objective of this study was to compare the efficacies of cooling systems of SSV and Cryotop methods for the cryopreservation of *in vitro* matured bovine oocytes in terms of oocyte survival fertilization and subsequent *in vitro* development of the resultant embryos.

4.3 Materials and methods

All reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise indicated.

4.3.1 Oocyte collection and *in vitro* maturation (IVM)

Collection and IVM of bovine follicular oocytes were performed as previously described (Imai et al., 2006). Ovaries from approximately 28-32 month-old Japanese Black heifers were collected at a local slaughterhouse and transported to the laboratory. COCs were then aspirated from small follicles (2–6 mm in diameter) using a 5 mL syringe connected with a 19 gauge needle. The maturation medium consisted of 25 mM Hepes buffered TCM199 (GIBCO BRL, Grand Island, NY, USA) and 5% calf serum (CS,

GIBCO BRL). COCs were washed twice with the maturation medium and cultured for 20 h in 600 μL droplets (in groups of 80-100/droplet) of the maturation medium covered with paraffin oil (Nacalai Tesque Inc., Kyoto, Japan) in 35-mm plastic dishes (Nalge Nunc International, Roskilde, Denmark) at 38.5 °C in humidified atmosphere of 5% CO_2 in air.

4.3.2 Oocyte vitrification

In vitro matured oocytes were partially denuded by a brief treatment with 0.1% (w/v) hyaluronidase followed by a gentle pipetting through a fine glass pipette. Equilibration and CPA treatments were performed by the methods previously described by Dinnyés and co-workers (Dinnyés et al., 2000). Briefly, the oocytes were washed three times in a basic medium (BM), which is 25 mM Hepes buffered TCM199 supplemented with 20% FBS (GIBCO BRL), and then treated with an equilibration medium, which is BM supplemented with 4% (v/v) EG, for 12 to 15 min at 38.5 °C. Groups of 5-10 equilibrated oocytes were rinsed three times in 20 μL droplets of a vitrification solution, which is BM supplemented with 35% (v/v) EG, 50 mg/mL polyvinyl pyrrolidone and 0.4 M trehalose (Sigma, T-0167) for 30 sec, and then either directly dropped with about 2 μL vitrification solution onto the chilled dry surface of an aluminum foil floating on LN_2 (SSV group) (Li et al., 2002) or placed on a sheet of a Cryotop (Cryotop group) and plunged into LN_2 (Kuwayama et al., 2000). Vitrified oocytes were warmed without storing by transferring microdrops/Cryotops into a warming solution (0.3 M trehalose in BM) at 38.5ºC. One to 2 min later, the oocytes were consecutively transferred for 1 min into 500 μL droplets of BM supplemented with each of 0.15 M, 0.075 M and 0.0375 M trehalose, respectively. They were washed three times in BM at 38.5 ºC and then returned into their original droplets of the maturation medium and incubated for an additional 2 h at 38.5 °C in humidified atmosphere of 5% CO_2 in air with satureted humidity.

4.3.3 Evaluation of oocyte viability

At 2 h after warming, the oocyte viability was evaluated by FDA staining according to the method previously described by Mohr and Trounson (Mohr et al., 1980). Briefly, oocytes were treated with 2.5 μg/mL FDA in PBS supplemented with 5 mg/mL BSA at 38.5 ºC for 2 min in a dark room and then washed three times in PBS supplemented with 5 mg/mL BSA and evaluated under an epifluorescence microscope (IX-71, Olympus, Tokyo, Japan) with UV irradiation using a U-MWIB3 filter with excitation wavelength of 460–495 nm and emission at 510 nm. Oocytes expressing a bright green fluorescence (Figure 4.1) were regarded as living ones and used in subsequent experiments.

4.3.4 *In vitro* fertilization (IVF)

IVF of surviving oocytes was carried out as previously reported (Imai et al., 2006). Briefly, frozen-thawed semen of a Japanese Black bull was thawed in a 37 °C water bath for 30 sec and then centrifuged in 3 mL of a 90% Percoll solution at 740 × g for 10 min. The pellet was re-suspended and centrifuged in 6 mL of sperm washing medium, which was Brackett and Oliphant (BO) solution (Brackett et al., 1975) supplemented with 10 mM hypotaurine and 4 U/mL heparin (Novo-Heparin Injection 1000, Aventis Pharma Ltd., Tokyo, Japan), at 540 × g for 5 min. Then the pellet was re-suspended with sperm washing solution and BO medium supplemented with 20 mg/mL BSA, to achieve the final concentrations of 3×10^6 spermatozoa/mL, 5 mM hypotaurine, 2 U/mL heparin and 10 mg/mL BSA. One hundred μL drops of this sperm suspension were prepared in 35 mm plastic dishes, covered with mineral oil and used as fertilization droplets. The oocytes were removed from the maturation medium, washed twice in BO medium supplemented with 10 mg/mL BSA, placed in the fertilization droplets (20 oocytes/droplet) and cultured for 6 h at 38.5°C in humidified atmosphere of 5% CO_2 in air with saturated humidity.

4.3.5 *In vitro* culture (IVC)

IVC was performed in 100 μL droplets of CR1aa medium (Rosenkrans et al., 1993) supplemented with 5% CS covered with paraffin oil. At the end of insemination, putative zygotes were completely denuded from cumulus cells and spermatozoa by gentle pipetting with a fine glass pipette in preincubated IVC medium. Fifteen to twenty five zygotes were placed in each culture drop and then cultured at 38.5 °C in a humidified atmosphere of 5% CO_2 in air with saturated humidity for 9 days. The day of IVF was considered as Day 0.

4.3.6 Evaluation of fertilization events

At 16-18 h after insemination some oocytes of each treatment groups were mounted on glass slides and fixed with acetic alcohol (acetic acid 1: ethanol 3) for at least 3 days. The oocytes were stained with 1% (w/v) orcein in acetic acid, rinsed in glycerol: acetic acid: water (1:1:3), and then examined under a phase-contrast microscope. Oocytes with a female pronucleus were considered as activated ones. Oocytes with a male pronucleus(i) and/or sperm heads with the contributing sperm tails were classified as fertilized ones (Figure 4.2). The presence of one female and one male pronucleus was considered as normal fertilization.

4.3.7 Evaluation of in vitro development

Embryo development was assessed by occasional viewing under a stereo microscope. Cleavage rates in each treatment group were recorded on Day 2. Since early developmental speed was reported to predict developmental competence (Holm et al., 1998) rates of embryos developing beyond the four cell stage were also recorded at this time. Rates of embryos developing to the blastocyst stage were recorded on Day 7, Day 8 and Day 9.

4.3.8 Blastocyst evaluation by differential staining of inner cell mass (ICM) and trophectoderm (TE) cells

Differential staining of ICM and TE nuclei in blastocysts was performed by the method previously described by Thouas and co-workers (Thouas et al., 2001) slight modifications. Briefly, blastocysts were simultaneously treated with 0.1 mg/mL propidium iodide (PI) and 0.2% Triton X-100 dissolved in Dulbecco´s phosphate buffered saline for 60 sec to permeabilize the membrane and stain the nuclei of TE cells. The embryos were then treated with 25 μg/mL Hoechst 33342 (Calbiochem, San Diego, CA) dissolved in 99.5% ethanol for 5 min, mounted on glass slides in glycerol droplets, flattened by cover slips and examined under UV light with excitation wavelength of 330–385 nm using an epifluorescence microscope (IX-71, Olympus, Tokyo, Japan). The nuclei of TE cells labeled by both PI and Hoechst appeared pink or red, whereas nuclei of ICM cells labeled only by Hoechst appeared blue. A digital image of each embryo was taken and cell numbers of both cell types were counted using the NIH Image J (v. 1.40) software (Abramoff et al., 2004). Total cell numbers were counted in all embryos. The numbers of ICM and TE cells were counted separately in those embryos which had clearly distinguishable populations of red and blue nuclei.

4.3.9 Experimental design

Four treatment groups were compared in this study: the Control group consisted of *in vitro* matured oocytes without exposure to cryoprotectants or cooling. To assess cryoprotectant toxicity, oocytes were exposed to equilibration and vitrification treatments as described above but without cooling and subjected to the rehydration (warming) procedure as well (CPA group). To compare cryodevice efficiency, *in vitro* matured oocytes were cryopreserved either by using Cryotops devices (Cryotop group) or SSV (SSV group). Their warming was also performed in the same way as described above.

Viability of oocytes in each group was compared 2 h after the treatments. Living oocytes were fertilized *in vitro* and resultant embryos were cultured *in vitro*. In each replication, a sample of 10 putative zygotes was selected randomly from each treatment group at 16-18 h after IVF to assess their fertilization status. The rest of the oocytes were subsequently cultured to compare the *in vitro* development and blastocyst cell numbers.

4.3.10 Statistical analysis

Data of embryo development, fertilization and embryo cell numbers were arcsine transformed and analyzed by one-way ANOVA using the KyPlot package (Ver. 4.0, Kyens Lab. Inc., Tokyo, Japan). Five replications of the experiments were performed.

4.4 Results

4.4.1 Effect of cooling device on viability of oocytes

The viability of oocytes selected by morphology was examined by FDA staining. The rates of living oocytes in non-vitrified, CPA and both vitrified groups were over 90% (Figure 4.1). There was no significant different in the rates of living oocytes after treatment among the Control, CPA, Cryotop and SSV groups.

4.4.2 Effect of cooling device on fertilization results after IVF of Surviving oocytes

As shown in Table 4.1, there was no significant different among the Control, CPA, SSV and Cryotop groups in the rates of fertilization and monospermy after IVF. The total fertilization rates of oocytes vitrified with Cryotop (78.0%) and SSV (64.0 %) groups were similar and did not significantly different from those of the Control (54.0±9.7) and CPA (62.0±11.1) groups. There was no significant different in the percentage of oocytes with normal fertilization among the Control (50.0%), CPA (58.0%), SSV (48.0%) and Cryotop (62.0%) groups. The rates of normal fertilization of oocytes in the Control

(36.0%) and CPA (40.0%) exposed groups were not significantly different from those of oocytes in Cryotop (34.0%) and SSV (36.0%) groups.

4.4.3 Effect of cooling device on *in vitro* development of surviving oocytes

As shown in Table 4.2, the cleavage rate in the SSV group was significantly lower compared to that of the CPA group but was not significant different from those of the Control and Cryotop groups. The percentages of embryos that developed beyond the 4-cell stage on Day 2 did not significant different between the Cryotop (41.9%) and SSV (26.4%) groups. however; the percentage in the SSV group was significantly lower than that of the Control group (45.1%). The rates of cleavage and embryo development to the blastocyst stage on Day 7, Day 8 and Day 9 were not significantly different between the Control and CPA groups. The blastocyst rates between Cryotop and SSV groups on Day 7 (6.1% vs. 8.0%, respectively), Day 8 (8.7 % vs. 9.8%, respectively) and Day 9 (12.8% vs. 10.3%, respectively) was not significant different, but they were significantly lower than those of the Control group (25.3%, 33.1% and 36.4%, respectively).

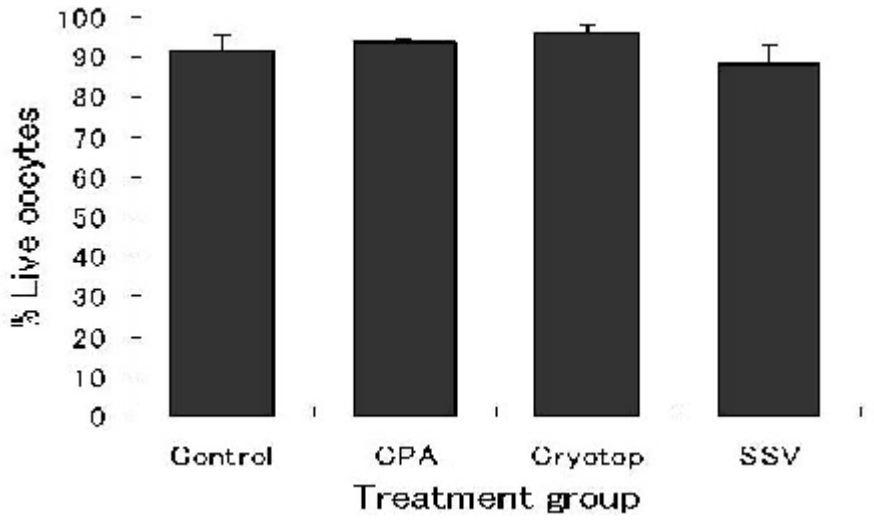


Figure 4.1 The viability of oocytes selected by morphology.

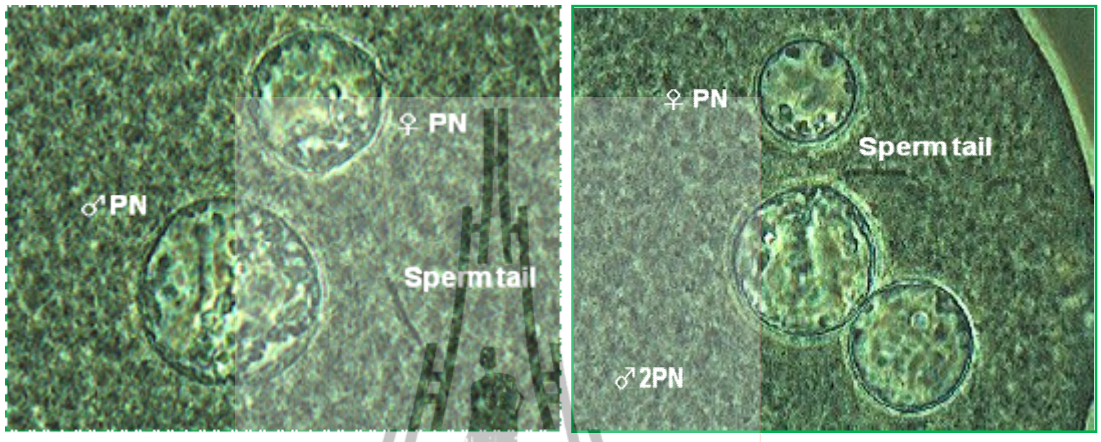


Figure 4.2 Activated Pronucleus; (A) Normal fertilization. (B) Abnormal fertilization.

Table 4.1 Fertilization status of oocytes at 18 h after IVF.

Treatment	Total oocytes examined	No. (%) oocytes		
		Fertilized	Pronuclear	Normal fertilization
Control	50	27 (54.0±9.7)	25 (50.0±8.9)	18 (36.0±11.2)
CPA	50	31 (62.0±11.1)	29 (58.0±9.1)	20 (40.0±8.3)
Cryotop	50	39 (78.0±8.0)	31 (62.0±6.6)	17 (34.0±8.1)
SSV	50	32 (64.0±7.4)	24 (48.0±8.6)	18 (36.0±12.0)

Five replications were performed. Data in parentheses are presented as % total ± SEM. Significant difference was not detected among the treatment groups at $P<0.05$ using one-way ANOVA. CPA = cryoprotectant; SSV = solid surface vitrification.

Table 4.2 *In vitro* development of control, CPA and vitrified oocytes after IVF.

Groups	Total oocytes cultured	Day 2 *		Oocytes (%) developed to the blastocyst stage on		
		Cleaved	> 4 Cell	Day 7	Day 8#	Day 9 #
Control	175	117 (65.9±10.9)[ab]	79 (45.1±3.2)[a]	44 (25.3±7.4)[a]	59 (33.1±7.3)[a]	65 (36.4±7.6)[a]
CPA	171	106 (61.3±5.9)[a]	80 (46.7±8.2)[a]	31 (18.1±2.6)[a]	40 (23.2±3.1)[a]	43 (24.8±3.9)[ab]
Cryotop	162	88 (53.2±9.4)[ab]	68 (41.9±7.3)[ab]	10 (6.1±3.7)[b]	14 (8.7±5.1)[b]	21 (12.8±5.0)[b]
SSV	140	55 (41.6±4.7)[b]	37 (26.4±6.6)[b]	13 (8.0±3.6)[b]	16 (9.8±5.0)[b]	17 (10.3±5.1)[b]

Five replications were performed. Data in parentheses are presented as % total ±SEM. [a,b)] Different superscripts in the same column indicate a significant difference at <0.05 (one-way ANOVA). *The day of IVF was considered as Day 0. # Cumulative values. CPA = cryoprotectant; SSV = solid surface vitrification.

Table 4.3 Cell numbers in blastocysts developed from control, CPA and vitrified oocytes.

Treatment	Total blastocysts evaluated	No. nuclei ± SEM		
		TE	ICM	ICM ratio
Control	52	65.5 ± 2.5	25.3 ± 3.9	32.7 ± 0.3
CPA	39	52.7 ± 4.9	30.5 ± 3.2	37.9 ± 0.5
Cryotop	17	46.1 ± 12.5	31.8 ± 2.8	37.5 ± 0.9
SSV	13	46.5 ± 17.0	27.4 ± 4.4	48.3 ± 1.8

Five replications were performed. Significant difference was not detected among the treatment groups at $P<0.05$ using one-way ANOVA. CPA = cryoprotectant; SSV = solid surface vitrification.

4.5 Discussion

Vitrification is a simple, rapid and cost-effective method for the cryopreservation of mammalian cells. Using this method cryopreserved cells are less likely to experience solution effects and intracellular ice formation compared to traditional slow cooling (Fahy et al., 1984). On the other hand, due to the high concentrations of cryoprotective agents the cells may be exposed to detrimental osmotic effects. Therefore, the period of exposure to CPAs before plunging into LN_2 is critical and should not be extended. High concentrations of CPA used for vitrification have been proven to be toxic to cells (Fuller et al., 2004; Kuwayama et al., 2005). However, in our study CPA treatment did not affect the survival and the development of oocytes suggesting that the present CPA treatment regimen is suitable for vitrification of MII bovine oocytes. MII oocytes are more difficult to cryopreserve than embryos due to the temperature sensitive metaphase spindle and its susceptibility to chilling injury (Shaw et al., 2000). Thus successful vitrification requires high concentrations of permeable cryoprotectants and a very rapid cooling rate which can

be provided by both Cryotop and SSV methods. (Kuwayama et al., 2000; Dinnyés et al., 2000). The Cryotop method applies a thin plastic sheet on which oocytes/embryos are placed with a minimum amount of vitrification solution before plunging into LN_2 (Kuwayama et al., 2000). On the other hand, the SSV method achieves a high cooling rate by using the combination of microdrops and improved heat exchange by direct contact with a dry metal surface cooled by LN_2 (Dinnyés et al., 2000). In this study, high survival and ferilization rates of vitrified oocytes were achieved by both the Cryotop and SSV methods which enabled their subsequent *in vitro* development after IVF. The high survival rates and normal morphology of vitrified-thawed bovine oocytes could have been achieved due to high rates of cooling and warming. Previous reports have shown that treatment with CPAs or cooling of MII oocyte may trigger biochemical reactions similar to those phenomena occurring during fertilization which can result in the hardening of zona pellucida and/or parthenogenetic activation of the oocyte in several mammalian species (Carroll et al., 1990; Somfai et al., 2007; Van et al., 1992; Larman et al., 2006; Matson et al., 1997; Tian et al., 2007). However, we observed similar fertilization, monospermy and pronuclear formation rates among the Control, CPA, SSV and Cryotop groups suggesting that the CPA treatment, both cooling methods and the warming regimen used in the present study did not cause such phenomena in MII bovine oocytes and allowed their normal fertilization by IVF. Also we observed similar rates of subsequent blastocyst formation on Day 7, Day 8 and Day 9 for oocytes vitrified by the Cryotop and SSV methods. Taken together these results indicated that the cooling strategies of SSV and Cryotop vitrification methods are equally effective for the cryopreservation of bovine IVM oocytes. In this respect both of these cooling methods can be recommended for cattle oocyte vitrification. The choice between the two methods may depend on the advantage and convenience of each method and the actual circumstances (e.g. the number of oocytes for cryopreservation) of the work. SSV is a very cheap method which enables the preservation of several microdrops of

vitrification solution in a short time, each containing a large number of oocytes. On the other hand, controlling the droplet size by this method requires extensive practice and there is a constant chance of oocyte loss since some oocytes tend to attach to the inner surface of the glass capillary during droplet formation. This method is therefore more suitable for the rapid cryopreservation of large quantities (hundreds) of oocytes/embryos. More precise control of the volume of the vitrified solution and oocyte numbers can be achieved by Cryotop vitrification and this method offers a convenient way of storage and handling after cooling. However, the numbers of oocytes that can be preserved on each Cryotop sheet is limited and the preservation of large numbers of oocytes by this method takes an extended period of time.

The cleavage and blastocyst formation rates of bovine MII oocytes cryopreserved by either the Cryotop and SSV methods in our study were higher than those reported by Roser and co-workers (Roser et al., 2008), using the Cryotop method. On the other hand, the cleavage and blastocyst formation rates of surviving vitrified oocytes in both the Cryotop and SSV groups were still reduced compared to those of the control and CPA groups. Similarly to previous studies on cattle and pig oocytes (Somfai et al., 2007; Dinnyés et al., 2000), treatment of MII stage oocytes with cryoprotectants without vitrification according to the SSV protocol did not influence their fertilization and in vitro development. Taken together with the high survival rates, this suggests that impaired developmental ability of vitrified oocytes was caused by sub-lethal damages which could be mainly related to the cooling and/or warming procedure. Such sub-lethal damages may include the disruption of meiotic spindle and other cytoskeletal elements (Chen et al., 2003; Albarracín et al., 2005), the damage and dysfunction of organelles such as the mitochondria or endoplasmic reticuli (Fuku et al., 1995; Rho et al., 2002; Lowther et al., 2009) or the degradation of cytoplasmic mRNA levels (Succu et al., 2008), which were reported to

occur in cryopreserved mammalian oocytes. Further improvements of the present vitrification protocols will be necessary to avoid such damages.

4.6 Conclusion

In conclusion, our data indicate that both the Cryotop and SSV vitrification methods could yield high survival rates of oocytes which could develop to the blastocyst stage after IVF. The cooling systems of Cryotop and SSV were proven to be equally effective to preserve IVM bovine oocytes in terms of their survival and subsequent developmental competence after IVF and IVC. However, further studies are necessary to improve the developmental competence of cryopreserved oocytes.

4.7 References

Abramoff, M.D., Magelhaes, P.J., and Ram, S.J. (2004). Image processing with image. **J Biophotonics International** 11: 36-42.

Albarracín, J.L., Morató, R., Rojas, C., and Mogas, T. (2005). Effects of vitrification in open pulled straws on the cytology of *in vitro* matured prepubertal and adult bovine oocytes. **Theriogenology** 63: 890-901.

Brackett, B.G., and Oliphant, G. (1975). Capacitation of rabbit spermatozoa *in vitro*. **Biol. Reprod.** 12: 260–274.

Carroll, J., Depypere, H., and Matthews, C.D. (1990). Freeze-thaw-induced changes of the zona pellucida explains decreased rates of fertilization in frozen-thawed mouse oocytes. **J. Reprod. Fert.** 90: 547-553.

Chen, SU., Lien, Y.R., Chao, K.H., Ho, H,N., Yang, Y.S., and Lee, T.Y. (2003). Effects of cryopreservation on meiotic spindles of oocytes and its dynamics after thawing: clinical implications in oocyte freezing-a review article**. Mol. Cell. Endocrinol.** 202: 101–107.

Chian, R.C., Kuwayama, M., Tan, L., Tan, J., Kato, O., and Nagai, T. (2004). High survival rate of bovine oocytes matured *in vitro* following vitrification. **J. Reprod. Dev.** 50: 685-696.

Dinnyés, A., Dai, Y., Jiang, S., and Yang, X. (2000). High developmental rates of vitrified bovine oocytes following parthenogenetic activation, *in vitro* fertilization, and somatic cell nuclear transfer. **Biol. Reprod.** 63: 513-518.

Fahy, G.M., McFralane, D.R., Angell, C.A., and Meryman, H.T. (1984). Vitrification as an approach to cryopreservation. **Cryobiology** 21: 407-426.

Fuku, E., Xia, L., and Downey, B.R. (1995). Ultrastructural changes in bovine oocytes cryopreserved by vitrification. **Cryobiology** 2: 139-156.

Fuller, B., and Paynter, S. (2004). Fundamentals of cryobiology in reproductive medicine. **Reprod. Biomed. Online.** 9: 680-691.

Ghetler, Y., Yavin, S., Shalgi, R., and Arav, A. (2005). The effect of chilling on membrane lipid phase transition in human oocytes and zygotes. **Hum. Reprod.** 12: 3385-3389.

Hochi, S., Terao, T., Kamei, M., Koto, M., Hirabayashi, M., and Hirao, M. (2004). Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. **Theriogenology** 61: 267-275.

Hochi, S., Fujimoto, T., Braun, J., and Oguri, N. (1994). Pregnancies following transfer of equine embryos cryopreserved by vitrification. **Theriogenology** 42: 483-488.

Holm, P., Shukri, N.N., Vajta, G., Booth, P., Bendixen. C., and Callesen, H. (1998). Developmental kinetics of the first cell cycles of bovine *in vitro* produced embryos in relation to their in vitro viability and sex. **Theriogenology** 50: 1285-1299.

Imai, K., Tagawa, M., Yoshioka, H., Matoba, S., Narita, M., Inaba, Y., Aikawa, Y., Ohtake, M., and Kobayashi, S. (2006). The efficiency of embryo production by ovum pick-up and *in vitro* fertilization in cattle. **J. Reprod. Dev.** 52: 19–29.

Kuwayama, M., and Kato, O. (2000). All-round vitrification method for human oocytes and embryos. **J. Assist. Reprod. Genet.** 17: 477 (Abstract).

Kuwayama, M., Vajta, G., Kato, O., and Leibo, S.P. (2005). Highly efficient vitrification method for cryopreservation of human oocytes. **Reprod. Biomed.** 300: 308 (Abstract).

Lane, M., Bavister, B.D, Lyons, E.A, and Forest, K.T. (1999). Containerless vitrification of mammalian oocytes and embryos. **Nat. Biotechnol.** 17: 1234-1236.

Larman, M.G., Sheehan, C.B., and Gardner, D.K. (2006). Calcium-free vitrification reduces cryoprotectant-induced zona pellucida hardening and increases fertilization rates in mouse oocytes. **Reproduction** 131: 53–61.

Li, X., Su, L., Li, Y., Ji, W., and Dinnyes, A. (2002). Vitrification of Yunnan cattle oocytes: work in progress. **Theriogenology** 58: 1253-1260.

Lowther, K.M., Weitzman, V.N., Maier, D., and Mehlmann, L.M. (2009). Maturation, fertilization, and the structure and function of the endoplasmic reticulum in cryopreserved mouse oocytes. **Biol. Reprod.** 18. [Epub ahead of print].

Martino, A.N., Songsasen, S., and Leibo, S.P. (1996). Development in to blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. **Biol. Reprod.** 54: 1059-1069.

Matson, P.L., Graefling, J., Junk, S.M., Yovich, J.L., and Edirisinghe, W.R. (1997). Cryopreservation of oocytes and embryos: use of a mouse model to investigate effects upon zona hardness and formulate treatment strategies in an *in vitro* fertilization programme. **Hum. Reprod.** 12: 1550–1553.

Mohr, L.R., and Trounson, A.O. (1980). The use of fluorescein diacetate to assess embryo viability in the mouse. **J. Reprod. Fertil.** 58: 189–196.

Rho, G.J., Kim, S., Yoo, J.G., Balasubramanian, S., Lee, H.J., and Choe, S.Y. (2002). Microtubulin configuration and mitochondrial distribution after ultra-rapid cooling of bovine oocytes. **Mol. Reprod. Dev.** 4: 464-470.

Rosenkrans, J.r. C.F., Zeng, G.Q., Mcnamara, G.T., Schoff, P.K., and First, N.L. (1993). Development of bovine embryos *in vitro* as affected by energy substrates. **Biol. Reprod.** 49: 459-462.

Roser, M., Dolors, I., Maria, T.P., and Teresa, M. (2008). Cryotop versus open-pull straw (OPS) as carriers for the cryopreservation of bovine oocytes: Effect on spindle and chromosome configuration and embryo development. **Cryobiology** 57: 137-141.

Shaw, J.M., Oranratnachai, A., and Trounson, A.O. (2000). Fundamental cryobiology of mammalian oocytes and ovarian tissue. **Theriogenology** 53: 59-72.

Somfai, T., Ozawa, M., Noguchi, J., Kaneko, H., Karja, N.W.K., Farhudin, M., Dinnyés, A., Nagai, T., and Kikuchi, K. (2007). Developmental competence of *in vitro* fertilized porcine oocytes after *in vitro* maturation and solid surface vitrification: Effect of cryopreservation on oocyte antioxidative system and cell cycle stage. **Cryobiology** 55: 115-126.

Succu, S., Bebbere, D., Bogliolo, L., Ariu, F., Fois, S., Leoni, G.G., Berlinguer, F., Naitana, S., and Ledda, S. (2008). Vitrification of *in vitro* matured ovine oocytes affects *in vitro* pre-implantation development and mRNA abundance. **Mol. Reprod.** 3: 538-546.

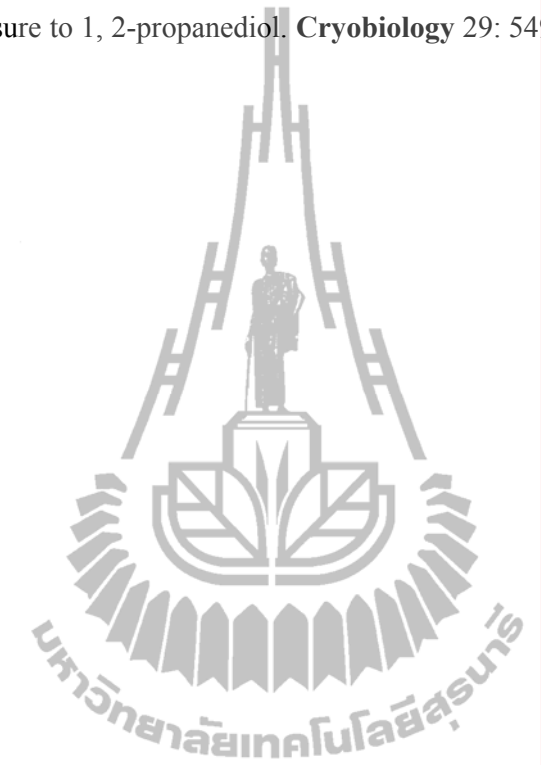
Thouas, G.A., Korfiatis, N.A., French, A.J., Jones, G.M., and Trounson, A.O. (2001). Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. **Reprod. Biomed. Online.** 3: 25-29.

Tian, S.J., Yan, C.L., Yang, H.X., Zhou, G.B., Yang, Z.Q., and Zhu, S.E. (2007). Vitrification solution containing DMSO and EG can induce parthenogenetic activation of *in vitro* matured ovine oocytes and decrease sperm penetration. **Anim. Reprod. Sci.** 101: 365-371.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobson, H., Greve, T., and Callesen, H. (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of

bovine ova and embryos**. Mol. Reprod. Dev.** 51: 53-58.

Van, der., Elst, J., Van, den., Abbeel, E., Nerinckx, S., and Van, S.A. (1992). Parthenogenetic activation pattern and microtubular organization of mouse oocyte after exposure to 1, 2-propanediol. C**ryobiology** 29: 549-562.



CHAPTER V

OVERALL CONCLUSIONS

Vitrification is a simple, rapid and cost-effective method for the cryopreservation of mammalian cells. Using this method cryopreserved cells are less likely to experience solution effects and intracellular ice formation compared to traditional slow cooling. No significant different was found among vitrified groups in maturation rate, cleavage and blastocyst rates after IVF. Similarly in solution exposure, after IVF, the cleavage and blastocyst rates among CB-exposed, FDA-exposed, CPA-exposed and CB+CPA-exposed were not different from the fresh control group. This study showed that GV stage bovine oocytes could be vitrified by CT and SSV methods with similar efficacy. Pretreatment with CB did not increase the maturation and embryo development of vitrified oocytes.

Study on MII stage bovine oocytes indicated that both the Cryotop and SSV vitrification methods could yield high survival rates of oocytes which could develop to the blastocyst stage after IVF. The cooling systems of Cryotops and SSV were proven to be equally effective to preserve IVM bovine oocytes in terms of their survival and subsequent developmental competence after IVF and IVC. However, further studies are necessary to improve the developmental competence of cryopreserved oocytes.

BIOGRAPHY

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Sripunya, N., Somfai, T., Inaba, Y., Nagai, T., Imai, K. and Parnpai, R. (2010). A comparison of Cryotop and Solid Surface Vitrification methods for the cryopreservation of *in vitro* matured bovine oocytes. **J. Reprod. Dev.** 56: 000-000.